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CIRRHOSIS AND OTHER HEPATIC LESIONS PRODUCED IN DOGS BY THYROIDECTOMY AND BY COMBINED HYPOPHYSECTOMY AND THYROIDECTOMY

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PLATES 1 TO 4

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Recent investigations have so focussed interest on the importance of dietary factors in the experimental production of fatty and cirrhotic livers that the significance of endogenous mechanisms in the pathogenesis of such liver disease has hitherto not been fully investigated. It is of interest, therefore, to report that fatty and cirrhotic livers can be induced in dogs deprived of both pituitary and thyroid glands even though they are made to ingest a diet rich in protein and adequate in all other respects.

The primary object of this paper is to present the pathological reactions encountered in the livers of a group of dogs subjected to thyroidectomy alone (T dogs) and to both hypophysectomy and thyroidectomy (HT dogs).¹ However, the pathological material acquired during these experiments also provided an opportunity for studying the pathogenesis of two histologically distinct forms of hepatic cirrhosis, namely that initiated periportally and that which originates in relation to the radicles of the hepatic veins. The occurrence of these two pathologically distinct forms of cirrhosis in animals treated in an identical manner was regarded as unusual enough to merit comment on their pathogenesis, with particular reference to the relationship between long standing fatty change and the onset of liver cirrhosis.

EXPERIMENTAL

The operative procedures employed for excision of the thyroid and pituitary glands, as well as the care and dietary treatment accorded the dogs both before and after operation, have been fully described elsewhere (1-3). In all dogs with two operations (HT) the hypophysis was

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¹ A preliminary report on the development of cirrhosis in the livers of dogs deprived of both pituitary and thyroid glands appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, 1943, 54, 170.

removed first, and the thyroid gland was resected at variable intervals thereafter. The diets consumed by the animals were adequate in regard to calories, proteins, salts, and vitamins (1-3). The dietary treatment of all dogs and the weight changes that occurred during their stay in the laboratory are summarized in Tables I and II. In all dogs with the two operations (HT), except HT11, appetite was lost after removal of the second gland. The exact caloric intake recorded for all dogs was maintained, when necessary, by forced feeding.

The completeness of hypophysectomy and the absence of hypothalamic lesions were established in all dogs at necropsy.

Thyroidectomized Dogs (T Dogs, See Tables I and III)

The most prominent lesion in the livers of this group of 8 animals was a pronounced fatty change. The liver cells toward the central veins were most severely affected, although in the majority of livers fat was visible throughout the lobules. Toward the portal tracts the fat tended to occur as multiple, varying sized, intracellular droplets, whereas in the centers of the lobules large single globules of fat virtually filled the cell and compressed the residual cytoplasm and nucleus to one side of the cell in the manner usually encountered in very fatty livers.

In addition to the fatty change, two other reactions of interest were noted. Firstly, in all of the animals an infiltration of plasma cells, lymphocytes, and polymorphs was observed, most frequently in the region of the central veins. This cellular infiltration, when marked and centrally located in the lobules, was often associated with a thickening and increased basophilia of the reticulum surrounding the individual enlarged fatty liver cells, apparently identical with that described by Lillie *et al.* (4) and by Costero and Barroso-Miguel (5). In some lobules elongated fibrocytic-cell nuclei were scattered in areas having thickened reticular fibers. This reaction, though not regarded as cirrhosis, can, in the light of the evidence presented below, be regarded as heralding a fibrosis. The second reaction worthy of record was the occurrence of fairly well marked edema and cellular infiltration around the sublobular divisions of the hepatic radicles.

Cirrhosis, of the severity described below, was not detected in the liver of any animal in this group of thyroidectomized dogs.

Dogs with Both Hypophysis and Thyroid Removed (HT Dogs, See Tables II and IV)

Fatty changes of varying severity were observed in the livers of 8 of the 9 dogs in this group. The livers of 4 animals were severely cirrhotic, while in 3 other animals early fibrosis of varying severity was present; in two of these 3 the normal lobular arrangement was for the most part retained, and consequently the changes were regarded as pre- or mildly cirrhotic rather than as frankly cirrhotic.

Fatty Change.—In 4 dogs the fat occurred as large globules in most of the

liver cells throughout the lobule. In the 4 animals in which some of the liver cells had escaped fatty metamorphosis, it was the cells toward the periphery of the lobules that were unaffected. Thus in Fig. 1 the dilated hepatic radicles are surrounded by enlarged fat-containing liver cells, while the zone of cells bordering on the portal tracts is rather mildly affected by the fatty change.

TABLE I
Dietary Treatment and Weights of Thyroidectomized Dogs

Dogs	Condition	Interval of observation	Dietary treatment*	Weight ranges		
				T2	T5	
		days		kg.	kg.	
T2 and T5	Before thyroidectomy	11	30 gm. meat and 5 gm. sucrose per kg. per day	7.9- 8.3	9.6- 9.9	
	After " "	1-65	Same as preoperative diet	9.0-10.0	11.0-12.7	
	" "	66-116	Alternate fast (5 days) and refeeding (5-12 days)	9.0-10.0	11.7-12.5	
	" "	117-236	15 per cent of preoperative diet	7.0- 9.0	9.0-11.7	
	" "	237-394	Alternate fast (5 days) and refeeding (5-11 days)	7.6- 8.1	9.9-10.4	
	" "	395-799	Same as preoperative diet	7.0-12.7	9.5-13.2	
T7	Before thyroidectomy	32	30 gm. meat and 5 gm. of sucrose per kg. per day	8.5- 8.6		
	After " "	387	Same as preoperative diet	8.6- 9.8		
				T12	T14	
T12 and T14	Before thyroidectomy	20	30 gm. meat and 5 gm. of sucrose per kg. per day	9.2- 9.6	12.4-13.0	
	After " "	1-20	Same as preoperative diet	9.7-10.1	13.2-14.6	
	" "	20-192	Alternate fast (5 days) and refeeding (5-7 days)	10.0-11.5	12.0-18.2	
	" "	193-460	Same as preoperative diet	11.2-12.5	11.4-18.2	
				T16	T17	T19
T16, T17, and T19	Before thyroidectomy	15	30 gm. meat and 5 gm. of sucrose per kg. per day	8.7- 9.0	8.1- 8.5	9.0-9.2
	After " "	300	Same as preoperative diet	9.0- 9.2	8.5- 9.4	9.0-9.8

* In addition to the dietary constituents listed above, each dog also received daily vitamin and salt supplements. A more detailed description of the treatment of these dogs will be found in previous publications (1, 2)

The severest fatty changes were detected in those livers classified as showing early fibrosis, and in these cases the cellular infiltration and fibrotic changes described and portrayed below occurred in and around the enlarged fatty liver cells in the central portions of the lobules (Figs. 2 to 5). The less severe fatty changes were present in those livers which were quite free of fibrosis, and also in the most severely cirrhotic livers.

Occasionally, in the livers free of fibrosis, round cell and polymorphonuclear

TABLE II

Dietary Treatment and Weights of Hypophysectomized-Thyroidectomized Dogs

Dogs	Condition	Dietary treatment*			Weight range
		Interval	Meat	Sucrose	
		<i>days</i>	<i>gm. per day</i>	<i>gm. per day</i>	<i>kg.</i>
HT3	Before hypophysectomy (H)	73	220	44	8.5- 9.1
	After H	58	220	44	9.0-11.0
	After H and thyroidectomy	97	220	44	10.6-11.2
HT6	Before hypophysectomy (H)	72	270	54	9.4-10.2
	After H	56	270	54	10.0-11.2
	After H and thyroidectomy	135	270	54	11.2-15.3
HT8	Before hypophysectomy (H)	75	250	50	8.0- 8.8
	After (H)	47	250	50	8.5-10.3
	After H and thyroidectomy	419	250	50	10.0-16.5
HT10	Before hypophysectomy (H)	69	260	52	8.6- 9.7
	After H	59	260	52	9.3-11.8
	After H and thyroidectomy	398	260	52	11.5-20.6
HT11	Before hypophysectomy (H)	8	180	36	6.0- 6.6
	After H	49	180	36	6.6- 7.5
	After H and thyroidectomy	374	180	36	7.3-12.5
HT18	Before hypophysectomy (H)	36	325	10	10.2-10.7
	After H	36	325	10	10.3-12.3
	After H and thyroidectomy	250	325	10	12.9-18.0
HT21	Before hypophysectomy (H)	37	260	10	7.7- 7.9
	After H	45	260	10	7.3- 8.0
	After H and thyroidectomy	217	260	10	7.6-14.5
HT22	Before hypophysectomy (H)	12	270	10	8.3- 8.5
	After H	45	270	10	8.1- 8.7
	After H and thyroidectomy	311	270	10	8.8-19.7
HT15	Before hypophysectomy (H)	35	300†	100	6.5- 6.8
	After H	47	300†	100	6.5- 9.1
	After H and thyroidectomy	68	300†	100	8.7-12.3
	" " " "	43	150†	50	12.0-12.1
	" " " "	71	54†	10	9.7-12.1
	" " " "	36	36†	6	9.0- 9.7
	" " " "	47	150†	50	9.0-11.5
	" " " "	25	0	0	9.0-11.5

* In addition to the dietary constituents listed above, each dog also received daily vitamin and salt supplements. A more detailed description of the treatment of these dogs will be found in a previous publication (3).

† Dog HT15 received throughout ground fish instead of lean meat.

TABLE III
Pathology of Liver of Thyroidectomized (T) Dogs

Dog	Sex	Time after thyroidectomy when sacrificed	Liver weight	Fatty acids in liver	Histological fat in liver	Hepatic fibrosis	Hepatic cirrhosis	Round cell infiltration in liver
		days	gm.	per cent				
T2	F	799	432	21.5	5+; droplets and globules; PSL*	0	0	Scattered foci
T5	F	799	431	22.7	2-3+; mainly globules; PSL patchy	0-2+; very patchy and mainly in subcapsular fatty lobules	0	PSL
T7	M	387	201	5.9	Small foci of PSL globules	0	0	PSL
T12	F	460	417	21.7	5+; PSL droplets and globules	0	0	PSL edema
T14	M	460	419	20.2	2-3+; mainly droplets, some globules; PSL and PP†	0	0	PSL edema
T16	M	300	253	6.6	2+; scattered globules; not in all lobes	1+; scattered but mainly PP not related to patchy fat	0	Scattered foci
T17	F	300	187	7.8	2+; scattered globules not in all lobes	1+; mainly in central fat and very patchy	0	Foci among fatty cells
T19	F	300	252	11.5	1-5+; globules, much variation in same section and in different lobes	0-3+; very patchy, mainly in very fatty subcapsular lobules, much variation in different lobes and even in same section	0-1+; very patchy varying markedly in same section and in different lobes	Focal and especially in subcapsular areas

Histologic grading of fatty change:

- 1+ = very small amounts mainly as droplets.
- 2+ = about one-quarter of lobule involved.
- 3+ = about half of lobule involved.
- 4+ = about three-quarters of lobule involved.
- 5+ = virtually entire lobule involved but not all liver cells with globules.
- 6+ = liver looks like adipose tissue; almost every cell with single large globule of fat.

Histological grading of hepatic fibrosis and cirrhosis:

- 1+ = present in scattered foci.
- 2+ = about one-quarter of lobules affected.
- 3+ = about half of lobules affected.
- 4+ = almost all lobes affected.

* PSL = around radicles of the hepatic veins, usually central or sublobular disjuncta.

† PP = periportal.

TABLE IV

Liver Pathology of Hypophysectomized-Thyroidectomized (HT) Dogs

Dog	Sex	Sacrificed		Liver weight	Fatty acids in liver	Histological fat in liver	Hepatic fibrosis	Hepatic cirrhosis	Remarks
		Time after hypophysectomy	Time after thyroidectomy						
		days	days	gm.	per cent				
HT3	F	42	97	450	13.7	3-4+; mainly globules; PP*	0	0	0
HT6	M	181	135	390	3.9	0	0	0	PP polymorphs, round cells, and edema. Marked sinusoidal distention
HT8	F	446	419	989	16.2	6+; some lobes are almost fat-free	3+; mainly PSL*; some PP	+	Fibrosis greater than HT10 and less than HT22
HT10	F	447	398	1455	30.5	5-6+; PSL	2+; PSL	+	Fibrosis not quite as advanced as HT8 and more so than HT22
HT11	F	423	374	285	12.5	3+; patchy PSL	3+; PP	2+	Plasma cells PP; PP fibrosis not related to centrilobular fat
HT15	F	357	310	375	52.5	1-5+; very patchy even in same section	4+	3+; very mixed PP interstitial and PSL	Well advanced cirrhosis makes determination of pathogenesis almost impossible
HT18	M	296	250	405	32.5	5-6+; globules; fat-free patches	4+; PSL	3+; mainly PSL; some PP	Later stage than HT8 and HT10 with early fibrosis of portal tracts
HT21	F	262	217	398	14.3	3-4+; PSL	4+; PSL	0	Pericentral fibrosis less advanced than HT10
HT22	F	356	311	412	11.2	5-6+; PSL. Lobes vary	4+; PSL	+	Fibrosis more advanced than HT8 but PP fibrosis not as marked as HT8 or HT18.

* Abbreviations as in Table III.

leucocyte infiltrations similar to those portrayed in Fig. 5 were observed around the sublobular veins.

Early Fibrous Tissue Reactions (Precirrhosis).—The uninterrupted series of changes detected in different livers revealed that the final cirrhosis commenced as the lesion described here as early fibrosis. Such early fibrotic reactions were identical in all the dogs, varying only in degree of severity. The initial changes are those portrayed in Figs. 3 and 5. It can be seen from these photomicrographs that in the regions of the central or sublobular veins the distance between the individual fat cells is distinctly increased. The tissue between the liver cells is faintly basophilic, this basophilia being due to an early thickening of the pericellular reticular fibers. These areas of thickened reticulum are clearly more highly cellular than is usual in such fatty areas, the increased cellularity being due to the presence of numbers of plasma cells, polymorphs, lymphocytes, and some fibroblasts (Fig. 3). For the most part, these inflammatory cells are distributed along the thickened reticulum, but numerous irregularly scattered foci of these same cells are also present. Large eosinophilic giant cells, usually with a single large contorted nucleus, are frequently scattered within, or in the neighborhood of, these cell foci (Fig. 5). The large number of these giant cells in the spleens of these animals (Fig. 6) suggests that these same cells may have infiltrated the liver by blood spread from the spleen. It must be added, too, that such foci of inflammatory cells are occasionally found scattered among those relatively fat-free liver cells towards the portal tracts. The most obvious site of infiltration of these inflammatory cells is around the walls of the central and sublobular branches of the hepatic veins, and it seems that these cells frequently occur here even when the reticular fibers around the fat cells are not yet obviously thickened (Fig. 5).

The thickening of the pericellular reticular fibers and the inflammatory cell infiltration are shortly succeeded by an accumulation of fibroblasts with long, vesicular, finely chromatic nuclei, and these cells, in turn, are followed by a further thickening of the reticulum, this time the result of collagen deposition. This combined series of reactions is regarded as early fibrosis of the centrolobular zones (Figs. 2 to 4).

With the progress of this early fibrotic lesion the reticulum thickens around the cells abutting on this primary zone of reaction, and subsequently the fibrosis becomes apparent even among the midzonal fat cells. The picture now seen reveals the presence of dense collagen with fewer fibroblasts but with new blood vessels in the central parts of the lobules. Radial extensions of these collagen fibers insinuate themselves among the fatty or non-fatty liver cells (Fig. 2). The centrolobular fibrous tissue of adjacent lobules now joins, forming a fibrous ring enclosing a centrally located portal tract and associated liver tissue of several lobules (Fig. 4).

With the contraction of this ring of fibrous tissue, the portal tracts are drawn nearer to one another and to the central zone of reaction, and some of the radiating fibrous tissue extends toward the centrally lying portal tracts

(Fig. 4). At this stage lobular distortion is initiated, and frank cirrhosis, which has obviously commenced around the central or sublobular veins, can be regarded as in progress.

Frank Cirrhosis.—As indicated in the introduction above, two pathologically distinct forms of cirrhosis were observed in the dogs which had both their thyroid and their pituitary glands resected. These two forms can be classified as (1) pericentral or perisublobular cirrhosis and (2) periportal cirrhosis.

1. *Pericentral or Perisublobular Cirrhosis:*—Both these terms are used here since the central vein may not be easily detectable, but instead a larger radicle of the hepatic vein forms the focus around which occurs the early fibrosis described above. The important point to be made is that *this is a cirrhosis commencing around the radicles of the hepatic rather than around the portal veins.*

With the progress of the early fibrosis described above, dense collagen masses form in the center of the lobule, the portal tracts are drawn closer, and are usually passively submerged in the progressing fibrosis (Fig. 7). At this stage the hepatic radicles draining the subcapsular lobules are obviously fibrotic, and the capsule itself is drawn in so that the liver surface has a dented appearance. Such pictures are indistinguishable from those described by Ashburn, Endicott, Daft, and Lillie (6) in the later stages of the cirrhosis produced in rats by diet and toxins. These workers demonstrated conclusively by injection of a carbon mass, that the affected veins were *hepatic* radicles.

At this stage reticular thickening followed by fibrosis commences in the *midzones* of the lobules and replaces the remaining liver cells. Such midzonal or intralobular fibrosis (Fig. 7) hastens the obliteration of those liver cells which are enclosed by the fibrous ring formed by the joining-up of the fibrosing hepatic radicles of neighboring lobules (Figs. 4 and 14). Even in the late stages of this form of cirrhosis, concentric fibrosis around the portal tracts is unusual (compare Fig. 4 with Figs. 9 and 10, and Fig. 13 with Fig. 14).

In the final stages of the centrolobular cirrhosis complete lobules are replaced by dense, fibrous scar tissue containing several portal tracts with their small atrophying bile ducts and several newly developed vessels (Fig. 8). Ultimately the hepatic architecture is so grossly distorted and the fibrosis is so diffuse that it becomes impossible to determine the site of origin of the fibrosis. However, in the livers obtained from the dogs in this experiment all stages of the reaction were detected. Even in the same lobe of one liver or in different lobes of the same liver, considerable variations were seen in the stages of evolution of the cirrhosis.

One of the striking features of the severely cirrhotic livers in this study was the complete absence of nodular hyperplasia and the rarity of bile duct proliferation.

2. *Periportal Cirrhosis:*—Since the fibrosis, in the two livers showing this lesion, clearly commenced in and around the portal tracts, this classification seems justified (see Figs. 9 to 13).

The initial reaction, detected around the smallest discernible portal radicles, is an accumulation of plasma cells and lymphocytes with an occasional polymorph around the tiny bile ducts (Fig. 12). This periductal cellularity, together with some thickening and edema of the periportal connective tissue, throws the affected tiny ducts, usually difficult of detection, into prominence (Figs. 9 and 10). In many instances the bile ducts themselves appear to be obliterated completely. Should they survive, the small portal tracts are made even more prominent (especially in preparations for reticulum or those stained with Mallory's connective tissue stain); this greater prominence is now due to the fibroblasts and collagen fibers being deposited concentrically around the portal tracts (Figs. 10 to 12). At this stage the liver, when stained with hematoxylin and eosin (Fig. 9), assumes an appearance similar to that portrayed by Watson and Hoffbauer (7) (*cf.* their Fig. IIB with our Figs. 9 and 10) and described by them as early cholangiolitic cirrhosis.

Progress of this periportal lesion is manifest by the extension of the fibrous tissue radially from the portal tracts into the lobules abutting on the affected portal tracts (Fig. 11). These tracts are now drawn closer to one another and to the radicles of the hepatic veins; intralobular fibrosis also occurs, and, as in the perihepatic vein cirrhosis described above, the lobules are eventually obliterated completely by dense scar tissue (Fig. 13).

Where extensive areas of liver are replaced by frank fibrous tissue, it is obviously impossible to determine the pathogenesis of the cirrhosis. However, the virtually complete absence in these dogs of nodular hyperplasia (usually encountered in severely cirrhotic livers) made the interpretation of the course of the lesions in our dogs very easy. Moreover, the graded series of reactions encountered in this experiment, in different animals and in different lobes of the same liver, left little doubt as to the pathogenesis of these two different forms of cirrhosis.

DISCUSSION

Our observations confirm for the dog the findings of Lillie *et al.* (4) and of Ashburn *et al.* (6) in the rat, that experimentally induced cirrhosis superimposed on a fatty liver usually commences and proceeds primarily around the radicles of the hepatic rather than of the portal veins.

In the dogs reported on here, this centrolobular cirrhosis occurred in animals with fatty livers. This finding would seem to add support to the contention that one of the important factors operating in the production of hepatic cirrhosis is a long standing fatty change. As early as 1938 Connor (8) stated, "There seems no room to doubt that long-standing fatty infiltration of the liver is a mechanical factor of great importance for the development of fibrous tissue. . . ." Recently Glynn (9) and Handler and Dubin (10) have supported Connor's view. This opinion is based on the fact that, no matter how the fatty liver is induced, whether by malnutrition, by toxins of chemical or bac-

terial nature (11-13), or by experimental pancreatectomy (14), cirrhosis is a frequent sequel.

From Tables III and IV it is clear that hepatic fibrosis of varying severity was detected in the livers of 4 thyroidectomized dogs and in 7 of the animals with the two operations (HT). In the latter group, frank cirrhosis of varying severity was diagnosed in 6 animals, while in only one of the thyroidectomized dogs (T19, Table III) was the fibrotic reaction sufficient to result in the lobular distortion which we have taken as an essential requisite for the distinction between hepatic fibrosis and frank cirrhosis. Comparison of Tables I and II reveals, furthermore, that in none of the thyroidectomized dogs was the fatty acid content of the liver greater than 22.7 per cent, whereas in several of the hypophysectomized and thyroidectomized dogs (HT) the fatty acid content of the livers was above 30 per cent and in one (HT15) it was 52.5 per cent. From a study of the chemically determined degree of fatty change, it is clear that, even among the HT dogs, no correlation can be established between the extent and severity of the hepatic fibrosis and the amount of fatty acid in the liver (Table IV).

It would seem from the findings in HT15 and HT18 (Table IV) that severe fatty change was associated with the most advanced cirrhosis. However, HT10, with a fatty liver of equal severity to that in HT18, had a much milder fibrosis and lobular distortion than the latter. These findings, together with the fact that HT22 (Table IV) with much less fat than either of those animals, had a fibrosis and cirrhosis more marked than did HT10 and almost as severe as in HT18, negate any attempt at correlation between severity of the fatty change and the onset and development of hepatic fibrosis.

Further, comparison of dog T12 (Table III) with dogs HT8, HT11, and HT22 (Table IV) or of dogs T12 and T14 (Table III) with dog T19 (Table III) makes even clearer our inability to predict, on the basis of the degree of fatty change, whether fibrosis or cirrhosis could be anticipated in histological sections of any liver.

Other evidence in support of the view that the fatty change is not alone responsible for stimulating fibrous tissue reactions in the liver is forthcoming from another study. As will be shown there, even the centrolobular cirrhosis described here has been observed in non-fatty livers (15). Moreover, of the two pathogenetically distinct forms of hepatic cirrhosis encountered in HT dogs, the centrolobular cirrhosis clearly occurred within the fatty liver lobules whereas the periportal fibrosis, observed in the livers of two animals, bore no detectable relation to the presence or absence or to the location of the fatty change. Thus in dog HT11 (Table IV) the fat content of the liver was relatively low, as judged by chemical and histological procedures, and the fibrotic reaction occurred in the non-fatty zones of the liver lobules (see Figs. 9 to 12.)

Whatever the final explanation of the lack of consistent correlation between

the fatty change in the liver and the onset of fibrosis in our dogs, we wish to suggest that the presence of long standing fatty change is not alone responsible for the onset of cirrhosis. The most that can be said at this stage is that severe fatty change around the central veins may facilitate the evocation of centrolobular cirrhosis.

Several other interesting facts emerge from the data accumulated in this study. Firstly, two pathogenetically distinct forms of fibrosis were detected in the dogs with both operations, even though they were subjected to the same experimental procedures. This rather unexpected finding indicates that the factors determining the localization of fibrous tissue reactions in the liver are still quite obscure.

Secondly, the incidence of severe fibrosis (amounting to cirrhosis) was far more frequent among the dogs with the two operations than among the animals deprived only of the thyroid. HT3 and HT6 (Table IV) were the only dogs with the two operations that did not develop hepatic fibrosis. This can probably be ascribed to the short time which had elapsed between the removal of the second gland (thyroid) and examination of the liver.

The removal of the hypophysis² would seem to have hastened the onset and rate of progress of fibrosis in the livers of our hypophysectomized-thyroidectomized dogs. Thus, severe fibrosis could be detected as soon as 250 days after the second operation in one dog (HT18, Table II), whereas in animals from which the thyroid alone was removed similar degrees of fibrosis were not present even after 300 days. Furthermore, the most severe fibrosis among the thyroidectomized dogs occurred in T19 (Table I) 300 days after removal of the thyroid, whereas a more severe and more extensive fibrosis was encountered within 250 to 300 days after thyroidectomy in dogs from which the pituitary had been previously removed.

The above considerations suggest that the presence of the pituitary in some manner inhibits cirrhosis-inducing factors from operating in the thyroidectomized dog.

Another fact emerging from this investigation is that both types of cirrhosis described here, which have usually been attributed solely to exogenous causes,

² Fibrous tissue proliferation has been shown to occur in the livers of hypophysectomized dogs by Graef *et al.* (16) and by Chaikoff *et al.* (17). The findings of the latter investigators differ somewhat from those of Graef *et al.* in that the development of the fibrous tissue was not preceded by an increase in the fat content of the liver. Graef *et al.* concluded that hypophysectomy had little if anything to do with the hepatic lesions and that the hypothalamic lesions seemed to have a pivotal rôle in the hepatic changes. Careful histological studies of the hypothalamic regions in our own hypophysectomized animals (17) by serial-section failed to reveal any evidence of damage to this important center. This fact, together with the absence of fat from the livers of our hypophysectomized dogs (17), indicates that the pathogenesis and possibly the etiology of the hepatic fibrosis in our hypophysectomized animals differed somewhat from those described by Graef *et al.*

can be induced by metabolic disturbances following surgically imposed endocrine deficiency. This evidence indicates that the endocrine disturbances known to occur in association with liver disease and chronic malnutrition deserve more attention than they have previously received in studies directed at the elucidation of the mechanisms involved in the production of hepatic cirrhosis.

SUMMARY

1. The reactions of the dog's liver to (a) thyroidectomy and (b) both hypophysectomy and thyroidectomy are described.

2. Fatty changes of varying severity were detected in 8 of the 9 hypophysectomized-thyroidectomized dogs, hepatic fibrosis in 7, and severe cirrhosis in 4 animals of this group.

Among the thyroidectomized animals histologically demonstrable fatty livers were present in all 8, mild fibrosis was observed in 4, while early and mild cirrhosis was diagnosed in only one dog.

3. Two pathogenetically distinct forms of cirrhosis were present in the livers of dogs with the two operations, namely (a) cirrhosis initiated and developing around the radicles of the hepatic veins, and (b) periportal cirrhosis.

Both forms of cirrhosis occurred in dogs that were subjected to the same experimental procedures, and both forms could be found in the same liver.

The pathogenesis of these two forms of cirrhosis is described.

4. The relation between fatty change in the liver and the genesis of fibrosis is discussed, and it is suggested that, while fatty change may facilitate the evocation of cirrhosis, this reaction on the part of the supporting and vascular elements of the liver is not solely dependent on the fatty change in the liver cells.

5. The relation between the endocrines and hepatic cirrhosis is discussed. Since cirrhosis was slight in the fatty livers of thyroidectomized dogs, whereas it was often advanced in hypophysectomized-thyroidectomized dogs, it is suggested that in the absence of the pituitary cirrhogenic mechanisms are facilitated in the dog.

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EXPLANATION OF PLATES

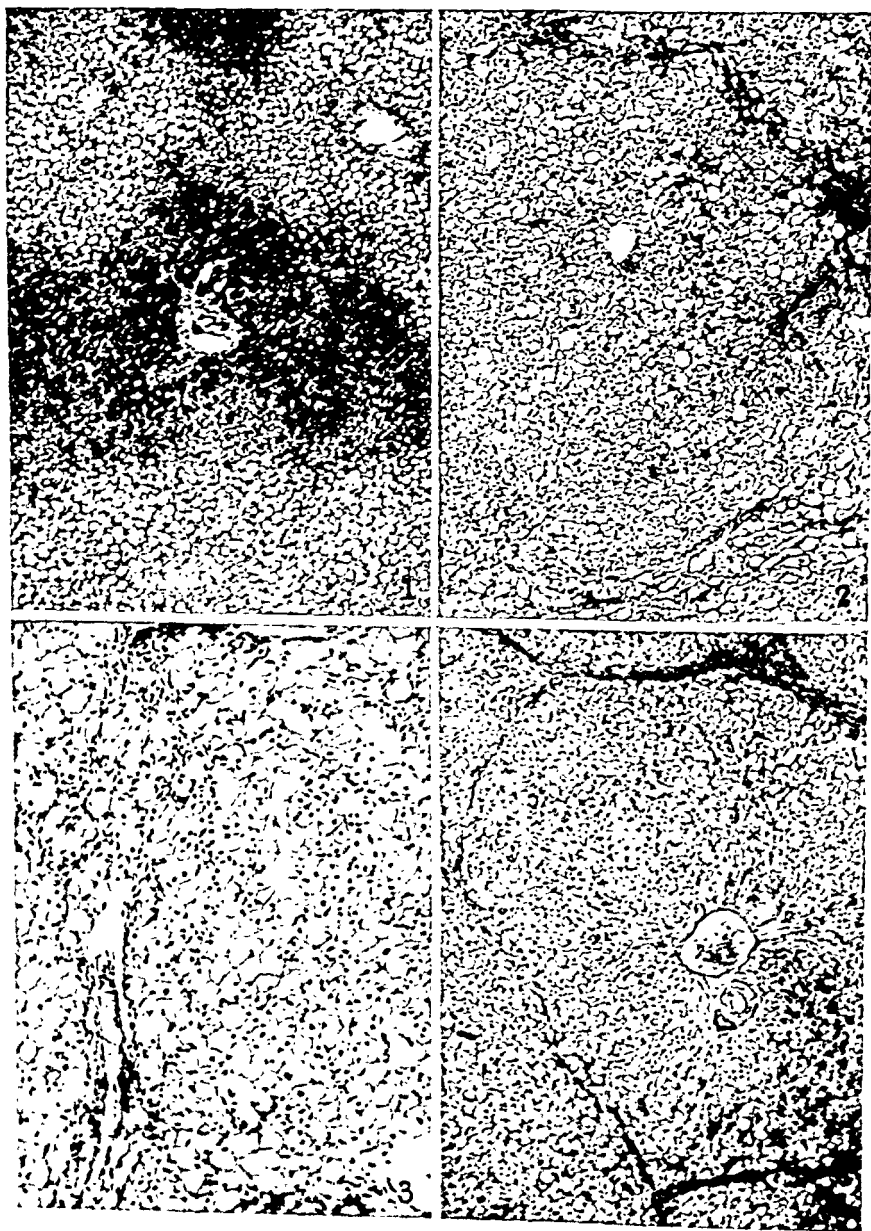
PLATE 1

FIG. 1. Section of liver of dog HT21 deprived of both pituitary and thyroid glands for 217 days. Note small portal tract in center surrounded by virtually fat-free liver cells. The three radicles of the hepatic veins are surrounded by very fatty liver cells indicating the centrolobular location of the fatty change. Note also some edema and cellular infiltration around the hepatic radicles. Hematoxylin and eosin. $\times 47$.

FIG. 2. Section of liver of dog HT10 deprived of both pituitary and thyroid glands for 398 days. Depicting early fibrotic changes (precirrhosis) among fatty cells in centrolobular area. Note that the small centrally located portal tract does not show any thickening or cellular infiltration. Hematoxylin and eosin. $\times 47$.

FIG. 3. Section of liver of dog HT10. Showing towards top and at left initial changes heralding precirrhosis in the centrolobular fat. The inter- and pericellular reticular fibers are thickened. There is an infiltration of round cells, polymorphs, and a few fibroblasts around and near the longitudinally sectioned hepatic radicle at left. The small portal tract at the bottom right of the picture shows no signs of fibrosis. Hematoxylin and eosin. $\times 98$.

FIG. 4. Section of liver of dog HT10. In this section the centrolobular fibrosis has progressed beyond that shown in Fig. 2. Collagen fibers have now been laid down in the region of the initial reaction within the centrolobular fat. In addition the fibrosis around the individual central or sublobular vessels is now becoming linked with that in the neighboring lobules (early cirrhosis). There is at this stage some early fibrotic reaction around the portal tract lying in the center of the area delineated by the linking of the perihepatic fibrous tissue bands. Hematoxylin and eosin. $\times 54$.



(Chaikoff *et al.*: Cirrhosis and other hepatic lesions)

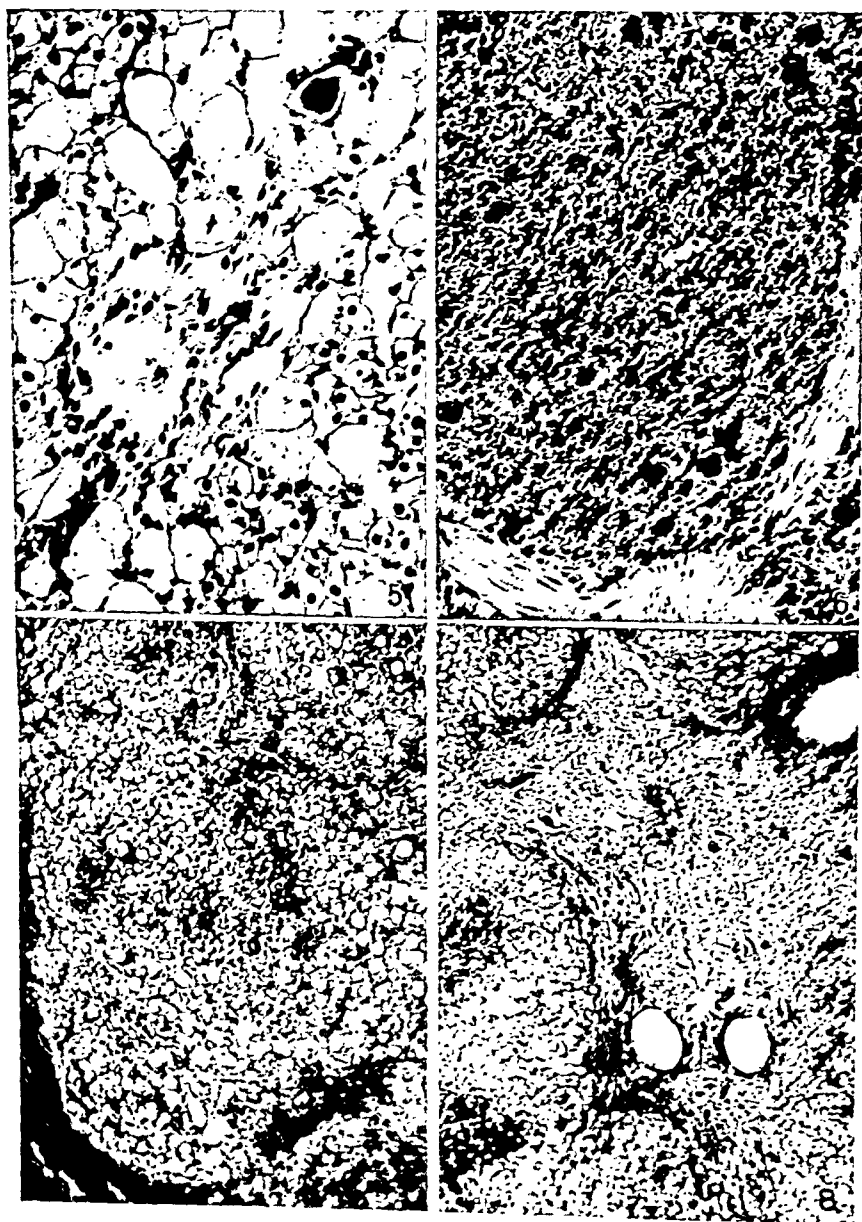
PLATE 2

FIG. 5. Section of liver of dog HT10. High power view of early reaction near central vein to show round cell and polymorph infiltration preceding early fibrosis. Large giant cells such as that seen toward the top right corner of this picture are commonly encountered in these early reactions. Hematoxylin and eosin. $\times 190$.

FIG. 6. Section of spleen of dog HT10. The large numbers of giant cells in the spleen of this animal, whose liver is portrayed in Fig. 5, and the fact that the splenic giant cells are morphologically identical with those in the liver suggest that the hepatic giant cells may have originated in the spleen. There is only one contorted nucleus in these giant cells. Hematoxylin and eosin. $\times 140$.

FIG. 7. Section of liver of dog HT18 deprived of both pituitary and thyroid glands for 250 days. A later stage in the progress of the centrilobular or perihepatic vein fibrosis. The early fibrosis depicted in Figs. 2 and 4 has progressed to dense, well marked fibrosis with lobular distortion (cirrhosis). Early fibrosis has begun above and to the right of the almost centrally located portal tract. This latter intralobular fibrosis begins within the lobule around the fatty liver cells in the same manner as depicted in the centrilobular region in the figures above. Hematoxylin and eosin. $\times 75$.

FIG. 8. Section of liver of dog HT18. Stage subsequent to that depicted in Fig. 7, showing terminal obliteration by fibrous tissue of an area (as shown in Fig. 4) delineated by the early linking of perisublobular fibrosis. Hematoxylin and eosin. $\times 47$.



(Chaikoff *et al.*: Cirrhosis and other hepatic lesions)

PLATE 3

FIG. 9. Section of liver of dog HT11 deprived of both pituitary and thyroid glands for 374 days. The small and medium sized portal tracts have become prominent by virtue of the periportal cellular infiltration and thickening. Note that this early periportal reaction is occurring despite the virtual freedom from fat of these portal tracts. This is regarded as an early stage in the pathogenesis of portal cirrhosis. The initial lesion here is clearly periportal. Hematoxylin and eosin. $\times 83$.

FIG. 10. Section of liver of dog HT11. Demonstrating the increase in collagen fibers around the small portal tracts of the liver depicted in Fig. 9. Mallory's connective tissue stain on another section from the same liver as that depicted in Fig. 9. $\times 41$.

FIG. 11. Section of liver of dog HT11. Later stage in the pathogenesis of periportal cirrhosis showing increased fibrosis around portal tracts with early extension of fibrous tissue among the liver cells abutting on the affected portal tracts. This is another lobe of the liver depicted in Figs. 9 and 10. Mallory's connective tissue stain. $\times 75$.

FIG. 12. Section of liver of dog HT11. The concentration of plasma cells and polymorphs around the bile duct rather than around the hepatic artery or portal vein in this small portal tract is clearly shown. From same liver as depicted in Figs. 9 to 11. Hematoxylin and eosin. $\times 330$.

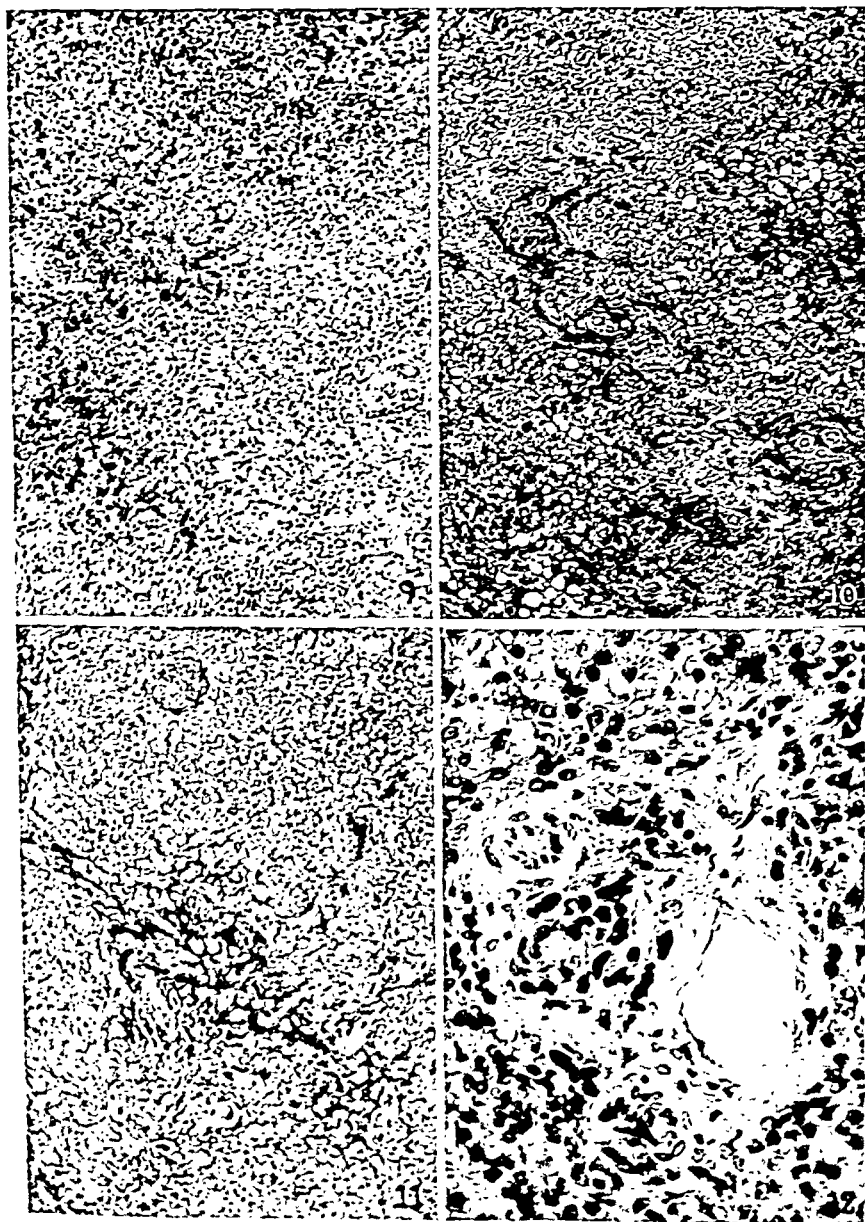
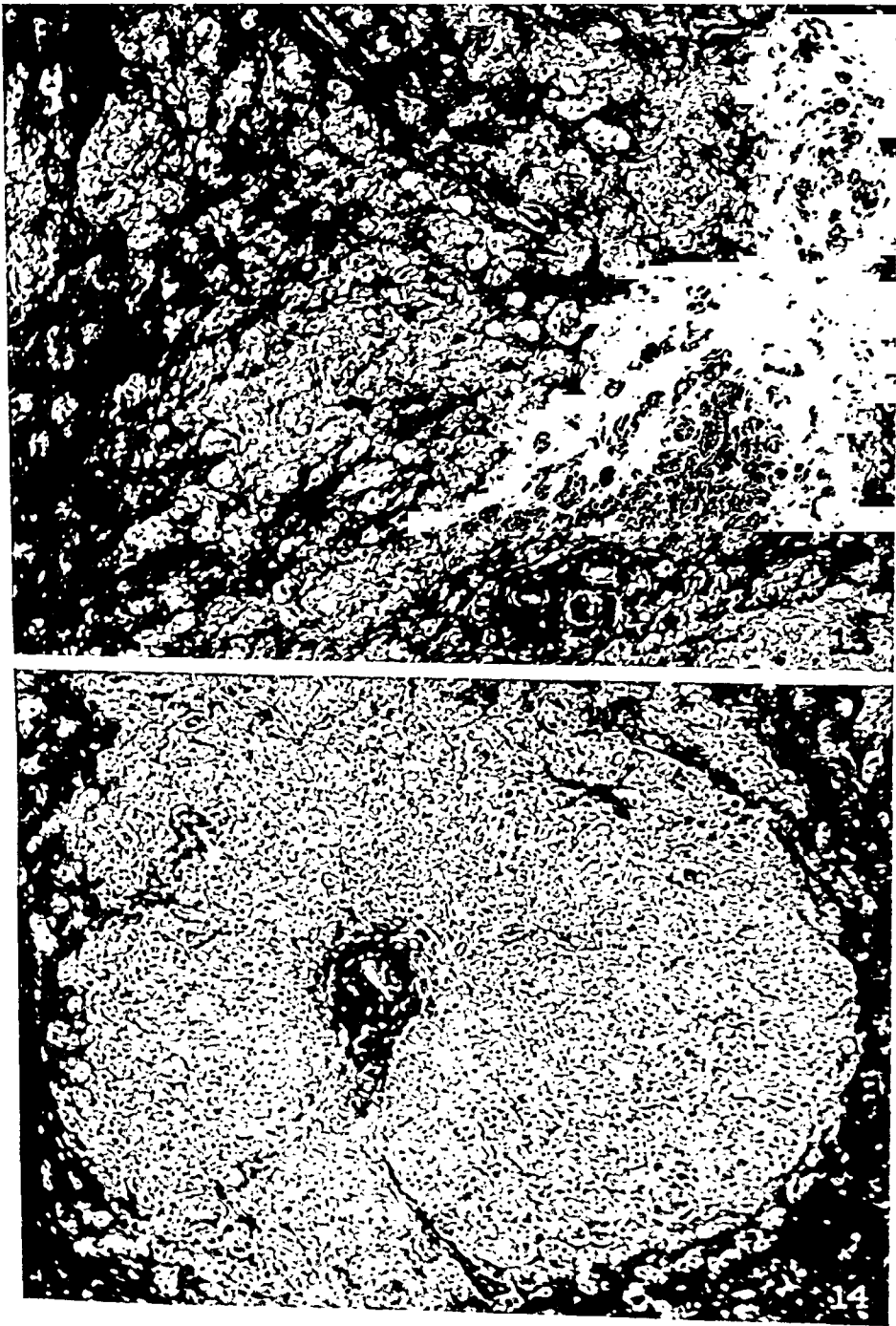
(Chaikoff *et al.*: Cirrhosis and other hepatic lesions)

PLATE 4

FIG. 13. Section of liver of dog HT11. Another lobe of same liver as that depicted in Figs. 9 to 12, showing here a later stage in periportal cirrhosis with severe involvement of parenchyma by the fibrous tissue extending from the densely fibrotic portal tracts seen on both the right and the left of the picture. Note, at bottom center, the dense concentric fibrosis around the small portal tract (a prominent finding in this type of cirrhosis) and compare with the relatively unaffected portal tracts, of similar size, in livers with the perihepatic radicle fibrosis in Figs. 2, 4, 7, and 14. Mallory's connective tissue stain. $\times 140$.

FIG. 14. Section of liver of dog HT15 deprived of both pituitary and thyroid glands for 310 days. Late stage in perihepatic radicle fibrosis showing dense fibrosis in laterally located hepatic radicles with extension of fibrosis into the parenchyma enclosed within fibrous "ring" (see fig. 4). Note that the centrally located portal tract shows relatively little reaction or thickening in this liver as compared with the periportal changes depicted in Figs. 9 to 13. Mallory's connective tissue stain. $\times 125$.



(Chaikoff *et al.*: Cirrhosis and other hepatic lesions)

A PARTICULATE BODY ASSOCIATED WITH EPITHELIAL CELLS CULTURED FROM MAMMARY CARCINOMAS OF MICE OF A MILK-FACTOR STRAIN*

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PLATES 5 TO 7

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The occurrence of mammary tumors in mice has been shown to be influenced by a transmissible agent, by an inherited tendency to develop breast cancer, and by hormonal stimulation of the gland tissue. Since the initial report of the existence of an extrachromosomal factor (1) and the demonstration by Bittner of its presence in the milk (2), many investigators have sought a definition of its mode of action, its transmission, and its character. Their results have shown the agent to have many of the characteristics of a virus (3-5). In order to define the nature of the agent more precisely, attempts have been made to study it with the electron microscope. At the time of this writing, we are aware of only two previous accounts of such efforts. Graff *et al.* (6) have examined the ultracentrifugate of milk of high-cancer and low-cancer strains of mice. In a brief note they report that the high cancer strain milk contains a "heavy particle" which "has virus-like dimensions." Passey *et al.* (7) made water extracts of desiccated normal and malignant breast tissue from mice of high- and low-cancer strains. In micrographs of material from high-cancer strains they found a particulate component about 200 Å in diameter which, they report, was not present in extracts of tissue from low-cancer strains. Both of these observations, the latter more than the former, are subject to the criticisms that the agent may be greatly altered by the preparation procedures and may be easily confused in microscopy with particulate elements present in the cytoplasm of all cells. A study of the cells themselves would be less subject to these criticisms and might, moreover, give some information on the mode of reproduction of the agent and its relation to the tissue cells.

Earlier reports from this laboratory have disclosed the suitability of cultured cells for electron microscopy (8) and have demonstrated that the method can be used not only for the study of new cytological detail (9) but can be applied as well to the identification of viruses in or on cells (10) and to the study of the special cytological features of malignant cells (11). It seemed worth while to

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† Fellow of the Finney-Howell Research Foundation.

use similar techniques in an investigation of the cells from mammary tumors of the mouse. The following is a report of the initial efforts.

Materials and Methods

The cells for study were grown from explants of both spontaneous and transplanted tumors. In all, four spontaneous and two transplanted tumors have been used. The former originated in female mice of the high-tumor strain C₃H, a strain in which the milk factor is known to be operative; the latter were fifth generation transplants of a spontaneous tumor¹ that also; arose in a C₃H mouse. All tumors were typical adenocarcinomas of the mammary gland that is to say they had the character of the growths known to be determined by the milk factor.

Cultures were prepared on formvar-coated slide inserts in roller flasks by methods described already (8). The explants were placed in shallow clots made up of equal parts of nutrient and chick plasma diluted 1:4 with Tyrode's solution. The nutrient was composed of 5 parts Tyrode's, 3 parts human cord serum, and 2 parts chick embryo extract. Each set of cultures was made with tumor tissue from a single animal, and successive sets were separated by intervals of 2 weeks or more. Different lots of cord serum, plasma, and embryo extract were used on each set. When, after a few days of culturing, small sheets of epithelial cells were obtained, the explants were removed, the remaining cells were washed briefly in a slow stream of Tyrode's (pH 7.4) and then placed in the vapor of osmium tetroxide for fixation. After periods over OsO₄, varying from 2 to 24 hours, the cells were mounted on screens and dried for electron microscope examination. All the micrographs were taken with an RCA (type E.M.U.) instrument.²

OBSERVATIONS

The culture conditions of the experiments seemed adequate, since within 2 days of culturing most of the explants showed surrounding sheets of epithelial cells. These continued to spread during the 3rd and 4th days, at the end of which time they were usually fixed. Occasionally the cells of an epithelial sheet were observed to cytolyze rather suddenly to be replaced later by a new growth.

A typical epithelial sheet is shown in Fig. 1. It is apparent that many of the cells are thinly spread and hence satisfactory for electron microscopy. Only small portions of a cell can be micrographed in any one exposure, so most of the illustrations include only a minute area approximating that outlined (A) in Fig. 1.

The electron microscope examination of preparations of the epithelial sheets had not gone far before it was observed that numbers of a small, apparently spherical particle were associated with some of the cells (Fig. 4). The characteristic density and morphology of these particles set them off from the normal cytoplasmic components (Figs. 5 and 7). In some cells they lay scattered in

¹ Designated as Law 916.

² The microscope was generously loaned to the project by Dr. R. M. Taylor, Director of the laboratories of the International Health Division of The Rockefeller Foundation.

small numbers (Figs. 4 and 5) while in others they literally packed the cell (Fig. 3).

Thus far in the material examined, the particles have been found associated only with epithelial cells or fragments of these cells. They can be seen in all parts of the cell and are not especially abundant nearer any particular component of it. Occasionally they appear to be within the endoplasmic strands and mitochondria (Figs. 4 and 5), and a few micrographs have shown them in the area of the nucleus (Fig. 3). It is difficult, however, to be certain whether they are actually within these structures or merely superimposed, the latter relationship seeming more probable in the case of the nucleus. Figs. 4 and 5 depict the more ordinary random association, and micrographs such as these show that the particles are generally situated in the ectoplasmic substance of the cytoplasm which, in these well extended cells, forms a thin layer between the membranes.

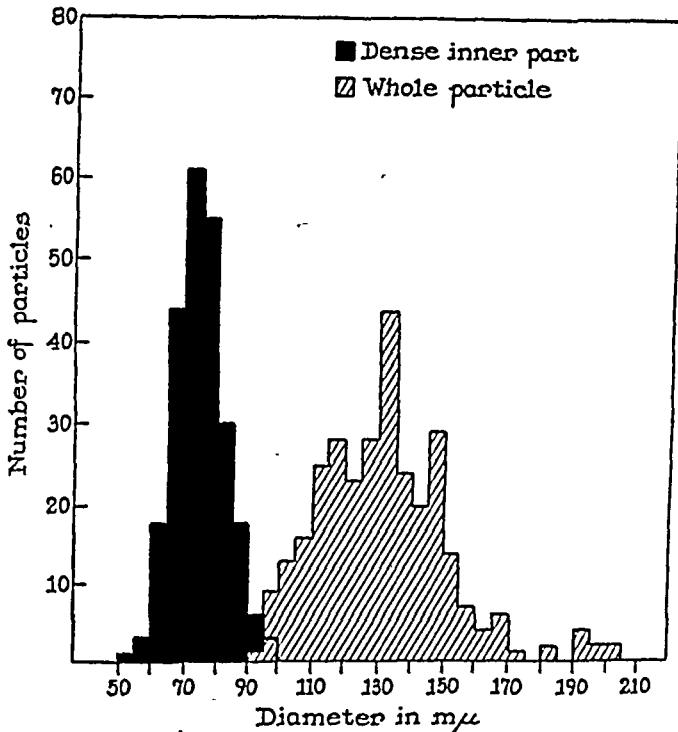
Small patches of cell membrane, sometimes with great numbers of particles on them, have been frequently found (Fig. 7). Evidently, while being washed prior to fixation, the cell proper was removed by the stream of Tyrode's and only portions of the membrane next to the formvar remained. The presence of the particles on such fragments indicates that in the intact cell they were located just within the cell membrane. Preparations of this sort are particularly valuable for good microscopy, since there is no overlying membrane and but little cytoplasm around the particles to scatter electrons and thereby reduce the definition. Similar fragments of cell membrane and other portions of the cell, probably products of natural cytolysis, have also been observed with associated particles, sometimes great numbers of them. Fig. 2 shows the particles definitely located on the cell surface. The significance of this observation is lessened, however, by the possibility that the cell may have ruptured in this region, releasing the particles shown.

The particles themselves are remarkably constant in character (Figs. 2 to 7). Their form appears to be spherical. Close examination of the micrographs (Figs. 4 and 6) reveals that most of them have a dark or dense center that is quite sharply defined and set off from a less dense, capsule-like periphery. This double structure is probably accentuated by OsO_4 fixation and may be consequent on the more osmiophilic properties of the central substance. In electron micrographs of gold-shadowed preparations it is possible in some cases (Fig. 5) to note that the central dense portion protrudes above a flattened border. This suggests that in desiccation the outer zone dries down more than the central part of the particle.

The diameter of the central, dense core is fairly constant from particle to particle and averages approximately 75μ (Text-fig. 1). The over-all dimension is more variable and averages about 135μ . Measurements on shadowed material indicate that the height of the dried particle is approximately one-half

the width. This is taken to mean that some flattening of the spherical form results from drying. The greater variation shown by the outside diameters (Text-fig. 1) is doubtless due in part to the fact that they are often poorly defined and not so accurately measurable. There is a suggestion in these findings as well that this portion of the particle is readily distorted, as if it were less rigid or viscous than the core.

The particles may occur singly, in pairs, or in clumps of all sizes. Some



TEXT-FIG. 1. Histogram to show size distribution of particles. It was constructed from 238 measurements of the diameter of the inside dense portion of the particle and 303 measurements of the over-all diameter. The values of these two average approximately 75 mμ and 130 mμ respectively.

groupings are of special interest. In Fig. 6 (A), for example, one can see at least two rosette arrangements formed by a single row of granules surrounding a central, unusually large particle. Clusters of four bodies, two large and two small, as in Fig. 7, are fairly common. It should be mentioned also that some of the unusually large particles look as if compound in structure; *i.e.*, composed of two, four, or more parts within a single capsule. The larger of such "encapsulated" clumps may have diameters almost double that of the average particle. Whether such special groupings of particles are stages in the reproduction of the typical form will have to be determined by further study. For studies of this type, the cultured material is especially favorable because

the particles are left relatively undisturbed and still associated with the tissue cell in which they are presumably multiplying.

Table I lists the origins of the material examined and shows the observed occurrence of the particles. It will be noted that in the preparations obtained from three out of six experiments none of the special sort described has been

TABLE I

Experiment	Date	Source of tumor	Age of tumor wks.	Mouse strain	Age of cultures when used days	Total no. screens	No. with particles	Quantity of particles
I	1947 Apr. 18	Law 916* Transplanted 5th generation 3 × 3 × 2 cm.	6	C ₃ H $\frac{1}{2}$	5-7	19	0	
II	May 14	Law 916* Transplanted 5th generation 3 × 3 × 2.5 cm.	11	"	7	12	1	Large numbers associated with the cells of only one small epithelial sheet
III	Aug. 29	Spontaneous 1 × 1 × 1 cm.	2	C ₃ H $\frac{1}{2}$ Lactating ♀	4	24	21	Isolated to abundant
IV	Nov. 24	Spontaneous 1 × 0.8 × 0.8 cm.	2	C ₃ H $\frac{1}{2}$	2-4	38	0	
V	Dec. 4	Spontaneous 1.5 × 1 × 1 cm.	6	"	3-8	14	0	
VI	Dec. 12	Spontaneous 1 × 0.7 × 0.7 cm.	5	"	3-6	46	25	Isolated to abundant

* Law 916, transplantable adenocarcinoma that arose spontaneously in a high-tumor strain C₃H mouse.

‡ Mammary tumor incidence approximately 90 per cent.

found. This does not prove that none was there, it means only that none was encountered during a moderately extensive examination of the screens. In the single preparation of transplanted tumor cells in which the particles were noted they were found only over a small area of the screen, in connection with a few cells. Screens from Experiment III (Table I), on the other hand, showed cells carrying great numbers of particles. Most of the illustrations were taken from this material. Particles were likewise more generally present

and abundant in the preparations of Experiment VI. Thus in these very limited studies the cells from spontaneous tumors derived from high tumor incidence C₃H mice have more commonly shown the granules than have those from transplanted tumors of similar derivation propagated in such mice.

DISCUSSION

These studies have disclosed the frequent presence in mouse mammary tumor cells of a particulate body, having a fairly uniform size, density, and morphology. Though similar in size, and possibly in other respects, to some normally occurring cytoplasmic granules (11), these particles are sufficiently different from the latter to give the impression of being special entities. Their uniform morphology, their association in closely packed clumps, as well as their irregular occurrence in tumor cells are especially significant as features distinguishing them from normal components of the cell. These same features make it seem probable that they are of extraneous origin and that they may be a virus.

The double structure shown by the particles cannot be explained as merely the image of a spherical body of uniform density. The latter, in an electron micrograph, would also show a relatively dark central portion, but its density should grade off gradually toward the periphery instead of suddenly altering to give a much lighter outer zone. Though evidence is lacking for any accurate interpretation of this finding, it does suggest the existence of a nuclear-like body surrounded by an envelope or capsule of different character. Electron micrographs of the viruses of equine encephalomyelitis (12), influenza A and B (13, 14), and vaccinia (15) have shown them to possess a similar complexity.

The variation in size recorded in the histogram (Text-fig. 1) is doubtless the reflection of a number of factors. Inaccuracy of measurement due to poor marginal definition will account for some of it; but the readily discernible differences in the diameter of the particles pictured make it clear that much of the variation is significant. As mentioned above, extremely large bodies have been seen that apparently consist of several small granules within a single "capsule." Possibly such large units are actually clusters of several small entities that have had their origin from a single particle. Conceivably, these small entities might enlarge to form separate particles of average size arranged around a primary one as in the rosettes shown in Fig. 6.

The most obvious, and for the moment, important question arising from these observations is whether or not the particles represent the milk agent of the mammary tumors. At present, there appears to be no simple and direct way of determining this; it will be necessary, instead, to gather evidence tediously through a comparison of tumor cells obtained from growths presumably carrying the milk agent with cells from the mammary tumors of agent-free animals. Such a study is in progress. Meanwhile, it may not be amiss

to consider the facts which favor identification of the particles with the milk agent. Numerous preparations of other cells from different species of animals, cultured in the same media as these mammary tumor cells, have not shown any particles of like appearance. It follows that they cannot have derived from the media. Secondly, in this connection, it seems significant that the particles are associated only with the epithelial cells of the cultures. Fibrocytes observed in the same preparations appear not to carry them. Other cell types from the mouse and from other species have been examined over the last 2 or 3 years, and no granules of precisely the same character have been encountered.

In view of these considerations and the fact that the cells were derived from tumors that arose in a high-tumor strain, it seems reasonable to assume tentatively that the particles are in fact the milk agent. In making this assumption we are aware of the possibility that the cells showing the particles may have come from tumors carrying an intercurrent virus. In this connection attention should be called to the tremendous difference in size between the particles reported upon here (1350 Å) and those observed by Passey *et al.* (7) in electron micrographs of material from mouse mammary tumors. In water extracts of tumors presumably carrying the agent these investigators found "approximately spherical particles about 200 Å in diameter" whereas in extracts of agent-free tumors nothing of the sort was observed. It is difficult to believe that this difference could be entirely a product of different preparation techniques. But further speculation may best be postponed until such time as Passey and his collaborators have tested the activity of their extracts and until the present studies of cultured cells have been extended to control material.

The electron microscope as a means for detecting intracellular inclusions is far from infallible, and unless the particles now under consideration are present in significant numbers or in clumps, they could be easily overlooked. This might account for their apparent absence in preparations made from some of the tumors, notably the two transplanted tumors studied. Yet it is possible that their scarcity in preparations from these latter represents a genuine scarcity. If so, the observation would tie in with some observations of Barnum, Ball, and Bittner (16) that the agent in transplanted tumors does not give as high a titre as in the spontaneous tumors.

It will be noted in Table I that in one set of preparations studied the particles appeared in the cells in tremendous numbers. The mouse from which these cells came was lactating at the time, and possibly this condition was attended by a multiplication of the agent. Conceivably also, the cells, if harvested an hour or two earlier, might have shown far fewer particles, whereas a little later they might have been destroyed.

Finally, it should be mentioned that a significant gain from these studies and our examination of normal cells, has been the experience needed for the

recognition of virus-like bodies associated with tissue cells. As one acquires the ability to discriminate the unusual or abnormal, the prospect develops that yet more pathological material can be examined with profit.

SUMMARY

Epithelial cells from spontaneous and transplanted mammary adenocarcinomas developing in high-tumor strain C₃H mice have been grown *in vitro* and studied with the electron microscope. In preparations from three out of six tumors, an unusual particulate body has been found associated with the cells. The particles appear to have a spherical shape and a double structure consisting of a dense center and less dense outer zone. The diameter of the central dense portion is fairly uniform from particle to particle, averaging approximately 75 m μ ; whereas the outside, whole particle diameter is more variable and averages about 130 m μ . From the micrographs it would appear that these peculiar virus-like bodies are situated chiefly in the ectoplasmic portion of the cell. They may occur singly, in pairs, or in clumps of varying sizes. Cells containing great numbers of the particles show signs of degeneration, and cell fragments are frequently encountered with many particles on them.

So far, the particles have been found only in association with the epithelial cells of the cultures. They are apparently not derived from the culture media. All in all the findings are consonant with the view that the particles represent the milk agent. Further evidence for or against this assumption is being sought from a study of cells from normal tissue and tumors demonstrated to be agent-free.

We are greatly indebted to Dr. Lloyd Law for his stimulating interest and the requisite material. It is a pleasure to acknowledge as well the technical assistance of Margaret Carr and Julia Hine.

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EXPLANATION OF PLATES

PLATE 5

FIG. 1. Photomicrograph of a typical sheet of epithelial cells grown from a spontaneous tumor explant. It illustrates the type of material that is suitable for electron microscopy. Small areas of approximately the size outlined (A) can be included in a single micrograph and the other figures are electron micrographs of such cell portions. The vacuoles are without significance in the present study. Taken from a 2-day-old culture, fixed in saline-formalin and stained with hematoxylin and cosin. $\times 200$.

FIG. 2. Electron micrograph of a portion of a cell margin showing particles attached to the edge of the cell and to pseudopodia projecting from the cell. At the bottom of the micrograph is the cell body. It is possible that the particles on the outside have been released from the cell through disruption of the membrane. It is to be noted that outside as well as inside the cell the particles possess a double structure which may be taken to indicate that the "capsule" is an integral part of the particle. Preparation made from 4-day-old culture of a spontaneous mammary tumor explant, fixed over vapor of OsO_4 24 hours. $\times 14,000$.

FIG. 3. Electron micrograph of a portion of a thick cell selected because it showed better than any other cell encountered in the preparation how gross the infection of particles may become. The large dark area outlined in white and shaped like a quadrant of a circular disc, which can be seen at the lower right-hand corner of the picture is part of the nucleus of the cell. The many particles which can be seen in this area may or may not be within the nucleus. Probably, as indicated by the definition of the image, they are between the cell and the nuclear membranes. Preparation from a 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO_4 . Shadowed with gold at an angle of 10° . $\times 12,500$.



(Porter and Thompson: Virus-like bodies in mammary carcinoma cells)

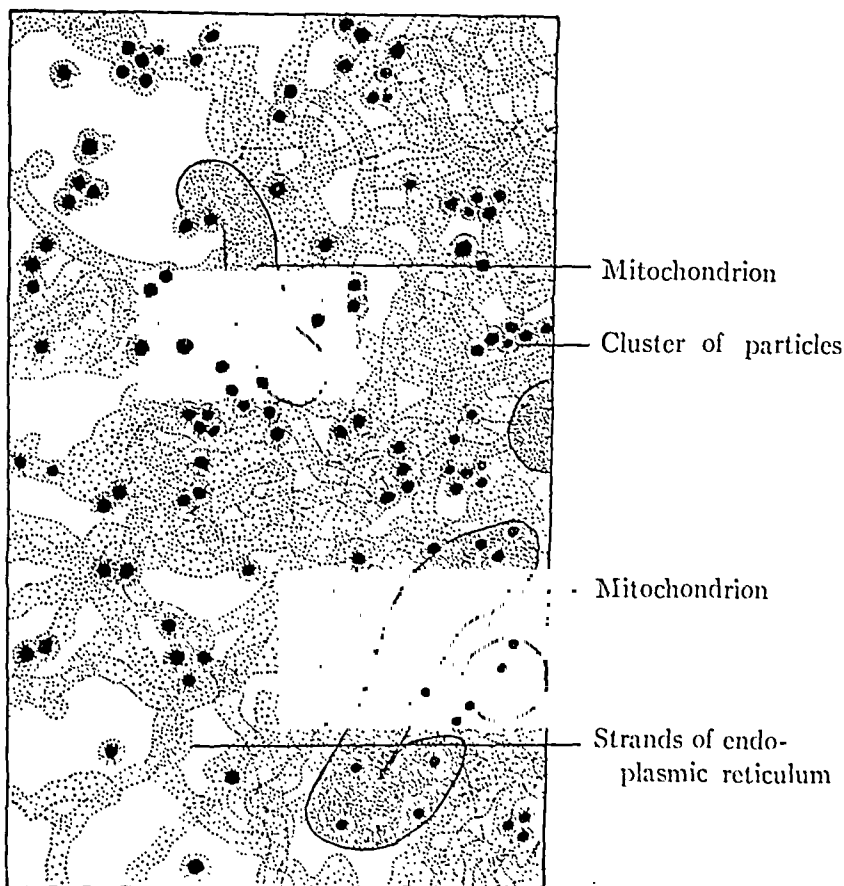
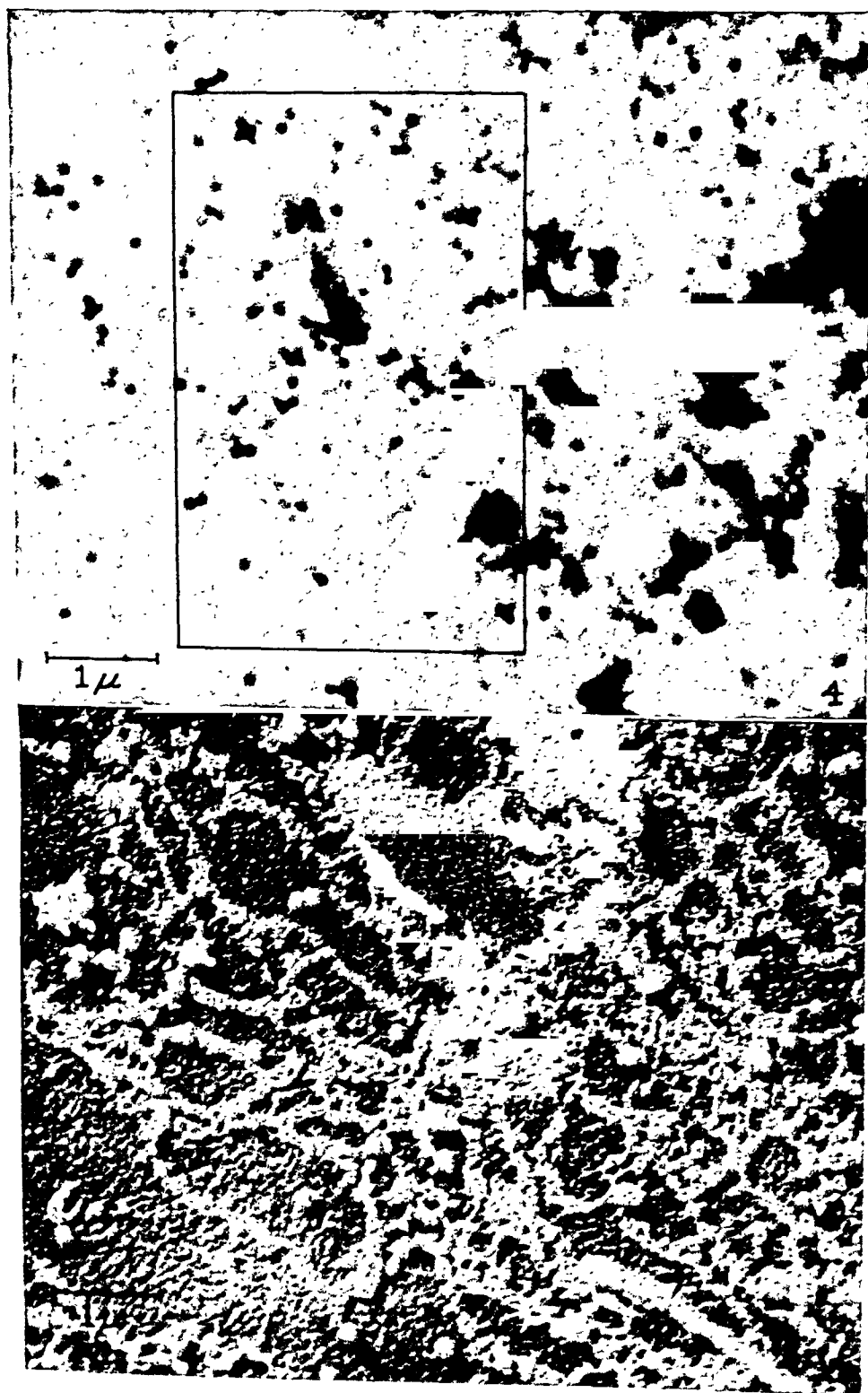


FIG. 4 a

PLATE 6

FIGS. 4 and 4 a. Electron micrograph of a small area of an epithelial tumor cell from a spontaneous C₃H mammary gland carcinoma growing in culture. Fig. 4 a is a descriptive diagram of a portion of it. The particles occur singly or in clumps of various sizes and are scattered about without apparent association with any particular cytoplasmic component. The relatively large mitochondria lie amidst strands of the endoplasmic reticulum. The upper cell membrane seems to be intact. Apparently the particles are situated in the ectoplasmic substance between the cell membranes. Some of them can be seen to show a central density and a peripheral "capsule." Preparation from a 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄. $\times 17,000$.

FIG. 5. Electron micrograph of a cell similar to that shown in Fig. 4, demonstrating by means of gold shadowing the three dimensional form of the dry particle contained in a cell. It can be seen that they have not dried down as much as the surrounding material and hence project up through the cell membrane, casting gold-free shadows. The mitochondria and strands of the endoplasm have flattened in drying. Some of the particles appear to be within the strands of endoplasm but may actually be superimposed. Preparation made from 4-day-old culture of a spontaneous mammary tumor explant, fixed 18 hours over vapor of OsO₄. Shadowed with gold at an angle of 10°. $\times 17,000$.



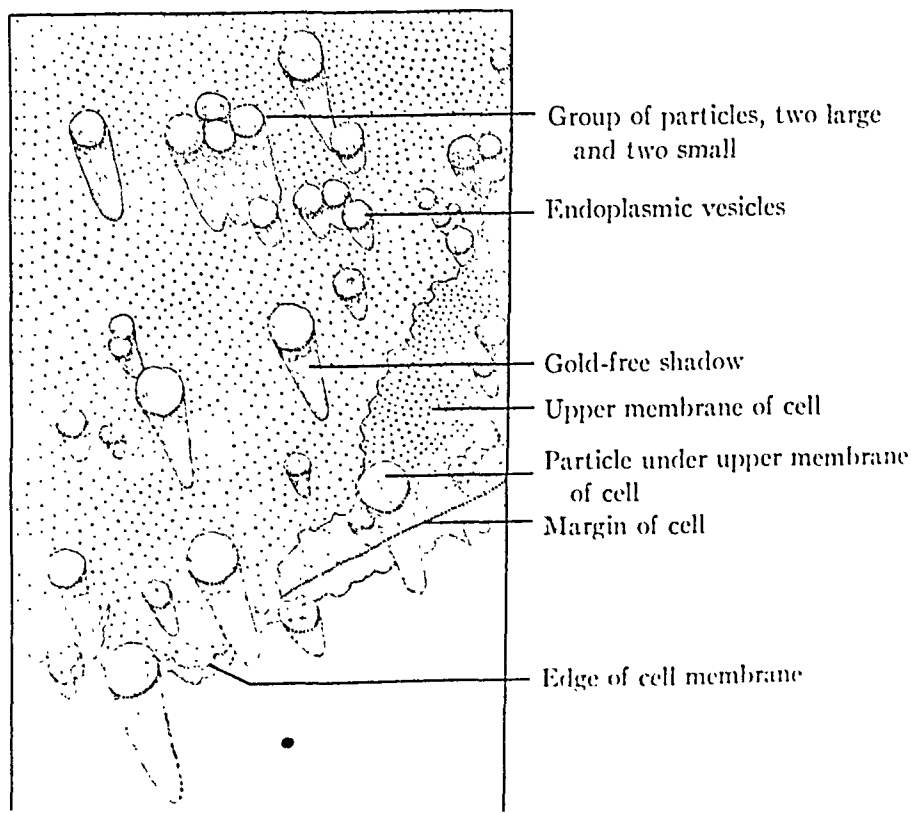
(Porter and Thompson: Virus-like bodies in mammary carcinoma cell)

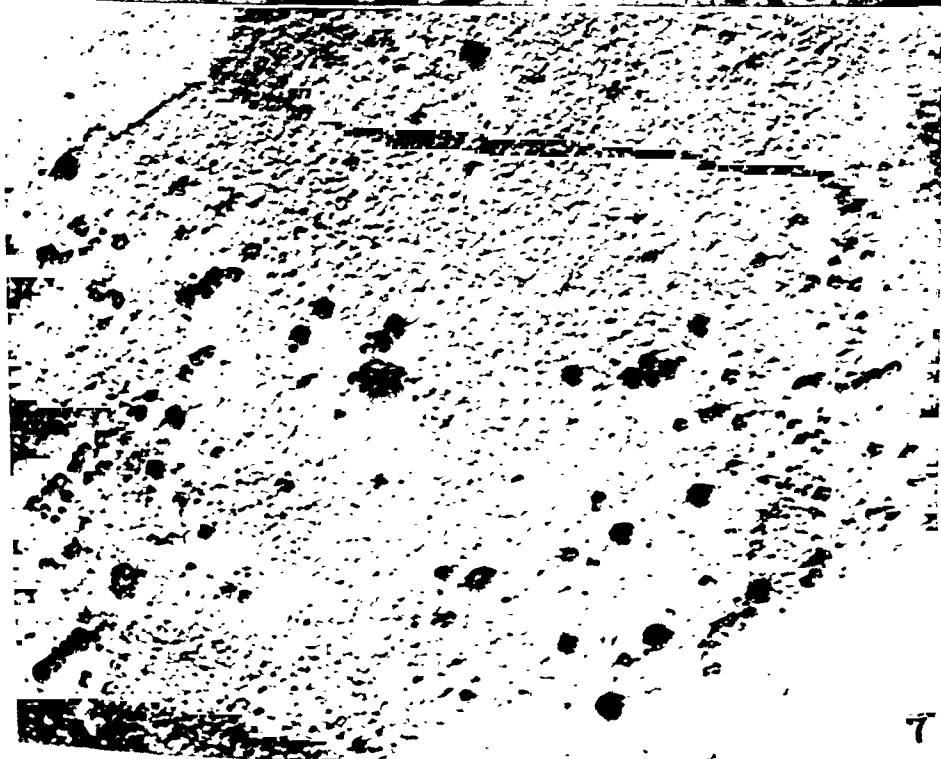
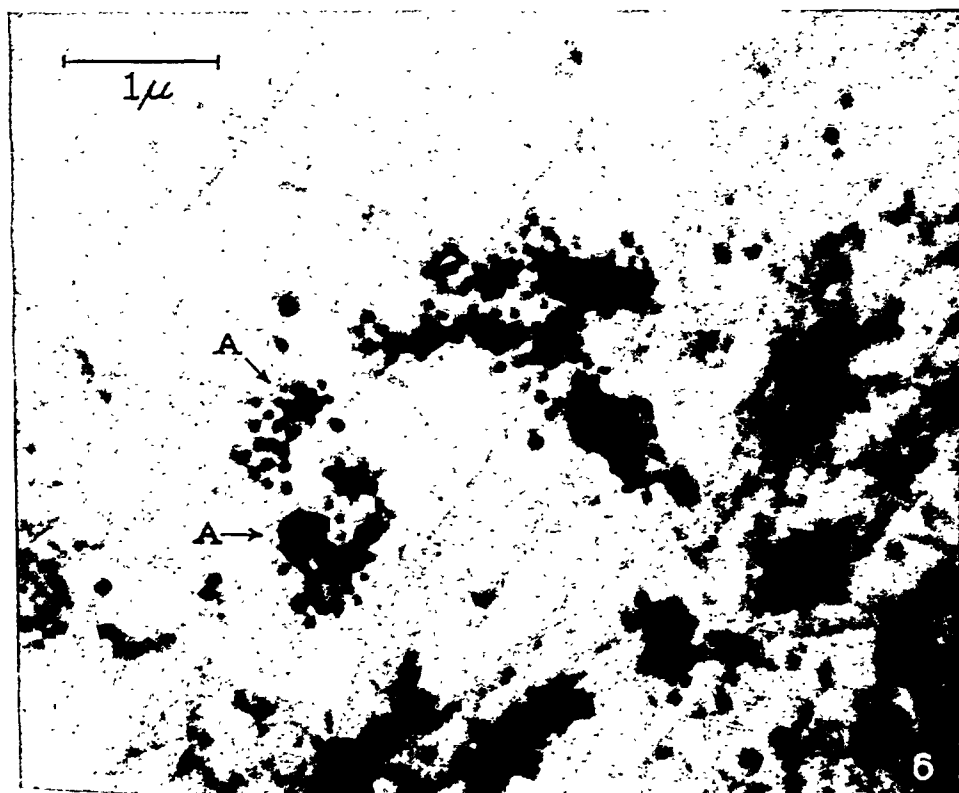
FIG. 6. Electron micrograph showing several clumps of virus-like particles. These were found in a cell of a small epithelial sheet from an explant of a transplanted tumor. It can be noted that the individual particle has a dense center and a less-dense periphery, and that there is a distinct variation in size. Curious rosette arrangements of some of the particles are indicated by (A). Preparation from a 7-days old culture, fixed over vapor of OsO_4 20 hours. $\times 21,500$.

FIGS. 7 and 7a. Electron micrograph of a cell membrane with particles attached and descriptive diagram of a portion of it. During the preparation of the cells for fixation, the superficial part of the cell was washed away, leaving merely a small portion of the membrane next the formvar. One margin of this fragment runs across the upper left-hand corner of the picture, and its other edge across the lower right-hand corner. The finely granular background is the inside surface of the cell membrane or to adsorbed macromolecules. The line with the deep shadow to the right is the real edge of the cell and the full thickness of the latter can be seen extending upward. In this triangular lighter area both membranes are present. The virus-like particles are the larger white bodies. They stand up approximately spherical and cast long shadows. The numerous smaller and flatter biscuit-shaped bodies are vesicles of the cytoplasmic endoplasm such as are commonly seen in normal cells.

It can be noted that the shadowed particles show considerable variation in size. The indicated arrangement of two small and two large particles has been observed in other micrographs.

Preparation made from a 4-day-old culture, fixed 18 hours over vapor of OsO_4 . Shadowed with gold at an angle of 10° . $\times 20,000$.





(Porter and Thompson. Virus like bodies in mammary carcinoma cells)

THE PHENOMENON OF IN VITRO HEMOLYSIS PRODUCED BY THE RICKETTSIAE OF TYPHUS FEVER, WITH A NOTE ON THE MECHANISM OF RICKETTSIAL TOXICITY IN MICE

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The rapid death of mice inoculated with yolk sac suspensions richly infected with the rickettsiae of murine typhus was first reported in 1940 (1). Shortly thereafter it was shown that similar suspensions of rickettsiae of epidemic typhus possess the same toxic property (2); and a serum neutralization technique based on this property was developed (3), which was highly specific in differentiating antibody of murine or of epidemic typhus origin (4). A similar toxic property has been demonstrated in yolk sac suspensions of *R. orientalis* of a single strain (Gilliam) only (5). The nature of the "toxin" has not been established, although it is apparently inseparable from the living rickettsial bodies (2). The mechanism by which death is produced in the mice has not been investigated.

Postmortem examination of mice dying after inoculation with suspensions of rickettsia-infected yolk sac suggested that blood clotting *in vivo* might play a rôle in the mechanism of death (6). In following this lead, a study was made of the effect of normal and infected yolk sac suspensions on heparinized whole blood of mice, guinea pigs, and rabbits. Contrary to what had been anticipated, it was observed that clotting occurred much more slowly in mouse blood mixed with infected yolk sac than it did in mouse blood mixed with normal yolk sac or with a combination of infected yolk sac and specific immune serum. Although this inhibition of clotting proved to be a reproducible phenomenon in the case of mouse blood, such was not the case with blood from the guinea pig and the rabbit. Instead, a different and equally interesting phenomenon was observed; *i.e.*, *in vitro* hemolysis of the heparinized rabbit blood by yolk sac preparations infected with typhus rickettsiae.

Normal yolk sac prepared in the same manner as the infected lots in no case showed hemolytic activity. The hemolytic principle of infected yolk sacs was recoverable from the sediment after centrifugation at speeds demonstrated to throw down most of the rickettsiae (4,000 to 5,000 R.P.M. for an hour). Finally, the hemolytic activity of these preparations was observed to be inhibited by the presence of immune serum. Further studies were indicated to determine the optimal conditions for the demonstration of hemolytic activity, with a view to

the investigation of the mechanism of specific hemolysis and of possible methods of application.

This report describes the hemolytic property of typhus rickettsiae, with observations on the mechanism of hemolysis and the utilization of this property in the development of a new serologic technique. Also described are brief *in vivo* experiments to determine the possible relation of the hemolytic property of rickettsiae to their toxic activity in mice.

Methods

Since description of the development of a technique for the demonstration *in vitro* of hemolysis caused by rickettsiae and of its inhibition with immune serum, constitutes an integral part of this paper, only certain basic procedures need be mentioned in this section.

The principal rickettsial strains employed were the Breinl (epidemic typhus) and the Wilmington (murine typhus), both of which had been maintained for a long but unrecorded series of passages in chick embryo yolk sac. Supplementary observations were also made with the South American strain (epidemic typhus) and the Gear strain (murine typhus), which were in relatively early passage (8th to 10th) in eggs. Yolk sac pools were prepared from eggs infected with rickettsiae by inoculation into the yolk sac (7) and harvested after 6 to 7 days' incubation at 35°C. The pooled yolk sacs were homogenized in a Waring blender as 20 per cent suspensions by weight in nutrient broth and were stored in sealed glass ampoules in a dry-ice chest until used. Only pools shown to be sterile by test cultures on nutrient agar and in glucose and thioglycollate broth, were employed. All dilutions of yolk sac indicated in the paper were made from the 20 per cent suspensions in nutrient broth, which were considered the original undiluted material.

Blood for the substrate for the tests was collected chiefly from the rabbit, by cardiac puncture. Heparin, to give a concentration in the blood of 1:10,000 to 1:20,000, or sodium citrate, to give a concentration of 0.1 per cent, was used when clotting was to be avoided. In addition to rabbits, the animals employed as a source of either blood or serum or for the demonstration of toxicity or infectivity of yolk sac pools were sheep, guinea pigs, eastern cotton rats (*Sigmodon hispidus hispidus*), and albino Swiss mice.

Pyrex tubes measuring 10 to 12 mm. by 70 mm. proved convenient for both hemolytic titration and serum inhibition studies. For incubation at any temperature above that of the room an ordinary bacteriological incubator adjusted to the desired temperature was used, unless otherwise stated. In a few cases where a short incubation period was employed, a water bath was used. Hemolysis was read on a scale ranging in degree from 4+ (very deep color all through the supernatant above the settled cells) through 3+, 2+, 1+, trace (tr.), and faint trace (ft. tr.), in the last of which only a minimum of hemoglobin had diffused into that supernatant immediately overlying the cells. Where end-points of hemolysis have been indicated they correspond to the dilution giving a reading of "tr."

Determinations of the toxicity of infected yolk sac were made by inoculating 0.25 ml. of serial twofold dilutions of suspended yolk sac into the tail veins of mice, and observing the number of mice dead after 18 to 24 hours (2). Lethality for cotton rats was demonstrated by inoculating similar yolk sac dilutions, also in 0.25 ml. volume, intracardially; the rats were then observed for a period of 10 days, during which time the number of deaths occurring was recorded (8). Infectivity determinations were made by inoculating 0.25 ml. volumes of serial fourfold dilutions of yolk sac intraperitoneally into cotton rats, which were challenged

intracardially 4 weeks later with a dose of between 2 and 4 LD₅₀. Rats surviving such challenge were considered to have been immunized by the original inoculum. Toxic, lethal, and infective titers were calculated by the 50 per cent end-point method (9).

EXPERIMENTAL

Factors Conditioning the Demonstration of Specific Hemolytic Activity

Volume Relationships.—The relative proportion of rickettsia-infected yolk sac dilution to red cell substrate does not appear to be of critical importance in preparations to be used for the observation of hemolytic activity. Identical hemolytic titers were obtained with either 2 parts of yolk sac to 5 parts of substrate or with equal volumes of each. The use of 5 parts of yolk sac to 2 parts of substrate resulted in a twofold reduction in titer. The volumes adopted for use were 0.2 ml. of yolk sac dilution and 0.5 ml. of substrate. Increase in these volumes up to 8 times without altering the proportions of the components had no effect on the titers.

Red Blood Cell Substrate.—The optimal composition of the red blood cell substrate was studied in considerable detail, and it is probable that the results obtained have some bearing on the mechanism of the hemolytic phenomenon, the nature of which remains obscure. The use of whole heparinized blood as the substrate, which was tried at first, proved unsatisfactory because the frequent occurrence of clotting in this material interfered with the observation of the degree of hemolysis. In an effort to overcome this difficulty, experiments were made with red cells plus saline and plasma or serum in varying proportions. The results of these experiments are summarized in Table I. Consistently, the maximum hemolytic titers have been observed when the cells constituted 25 per cent or more of the substrate, while the use of fewer cells resulted in proportionately lower titers. A corollary to this observation of the interdependence of red cell concentration and hemolytic titer is the fact that hemolysis of all the cells in the substrate has never been observed; decrease in the concentration of cells in an effort to obtain complete hemolysis has led instead to a decrease in degree of hemolysis. Thus, the concentration of red cells present in the substrate appears to be a critical factor in hemolysis.

The composition of the substrate, apart from the concentration of the red cells, seems to be of minor importance. Plasma was ruled out for routine use because of the frequent occurrence of clotting when it was employed. The substrate adopted was 25 per cent red blood cells, 25 per cent serum, and 50 per cent saline. This combination was decided upon because results of several experiments, such as Experiments 3 and 4 listed in Table I, suggested that it yields slightly higher and more consistent titers. Sodium citrate may be substituted for heparin as an anticoagulant for use in collecting the red cells. In some of our experiments we have employed as the substrate defibrinated blood diluted with an equal volume of saline. This has yielded satisfactory results at times, but cells prepared in such a way may undergo spontaneous hemolysis, due perhaps to physical injury to the cells during the process of defibrination.

The animal used as donor of the red blood cells employed in most of our studies has been the rabbit. In a few tests sheep cells were employed with essentially similar results; but the lack of a source for aseptic bleeding in the case of this animal caused us to discontinue its use, since its red cells offered no apparent advantage over those of the rabbit. As a matter of interest, the cells of other animals were investigated for their susceptibility to the hemolytic action of typhus rickettsiae. Erythrocytes of the mouse, cotton rat, and guinea pig all proved completely resistant to hemolysis when suspended in dilutions of either their own or rabbit serum.

The use of sera from species other than the rabbit as a component of the substrate was

explored. This was of practical importance since such sera were to be employed in studying the inhibition of the hemolytic activity by specific immune sera. It was observed that fresh guinea pig or cotton rat serum occasionally caused some hemolysis of rabbit cells, but this was generally found to be reduced or eliminated by heating the serum at 56°C. for 1 hour. Such heated serum when present in relatively large amount (25 per cent of the red cell substrate) in general led to slight reduction in the hemolytic titer as compared with that obtained with rabbit serum; but this reduction was not of sufficient degree to invalidate its use in small amount for serological testing. It was further shown that similar heating of the rabbit serum

TABLE I

Relation between Hemolytic Titer of Infected Yolk Sac Pools and the Composition of the Red Cell Substrate

Rickettsia strains used to infect yolk sac*	Experiment No.	Composition of substrate Per cent of				Hemolytic titer (1:x)
		Plasma†	Serum§	Saline	Red cells‡	
Wilmington	1	25		50	25	128
		25		65	10	64
		25		70	5	32
	2	50		25	25	64
		25		50	25	64
		10		65	25	64
Breinl	3		75		25	32
			25	50	25	64
				75	25	64
				90	10	8
	4		25	50	25	64
				75	25	32

* Dilutions with saline.

† Plasma and red cells obtained from heparinized rabbit blood.

§ Serum obtained from rabbit blood drawn without anticoagulant.

|| Titer read as dilution of 20 per cent infected yolk sac in which a trace of hemolysis was observed.

component of the usual substrate did not affect the demonstration of the hemolytic activity of suspensions of infected yolk sac.

Temperature and Time of Incubation.—The hemolytic reaction was found to be favored by moderately warm temperatures. In a typical experiment with yolk sac infected with rickettsiae of epidemic typhus, hemolysis was negligible when the mixtures of yolk sac and substrate were kept for 24 hours at 4°C., whereas after the same period at room temperature or at 35°C. the hemolytic end-points were 1:128 and 1:256 respectively. From successive readings made after 2, 4, and 18 to 24 hours of incubation at 35°C., it was also evident that hemolysis was not instantaneous but was relatively slow moving and progressed even after 4 hours' incubation, since the end-points observed at the 4-hour interval were one or two dilutions lower (two to four times less) than those observed in the same titration series after

18 to 24 hours. The significance of these findings in relation to the study of the reaction will be discussed subsequently. A time period of 18 to 24 hours at 35°C. has been selected as giving the optimal hemolytic titers. It is, of course, necessary to avoid bacterial contamination of all reagents in the performance of a test requiring such a prolonged incubation at a warm temperature.

The Hemolytic Principle

Hemolytic activity has been demonstrated in yolk sac preparations of all the strains of murine and epidemic typhus rickettsiae tested. It has also been found in a liver suspension from cotton rats infected with the Gear strain of the murine typhus rickettsia and in fresh peritoneal washings from cotton rats infected with the Wilmington strain of this rickettsia. Titers obtained varied from pool to pool of infectious material, but the majority of yolk sac pools rich in rickettsiae showed end-points of from 1:32 to 1:128. Preparations of several strains of *R. orientalis* (the organism of tsutsugamushi disease), including the Gilliam strain, have never exhibited any hemolytic activity.

Stability of the Hemolytic Principle.—One of the early observations on the nature of the hemolytic principle was its marked instability. Exposure of active yolk sac suspensions to 0.5 per cent formalin, which renders rickettsiae non-infective, was also shown to destroy the hemolytic principle completely. The far less drastic treatment of exposure to moderate temperatures was found to be accompanied by partial or complete loss of activity. The results of the studies on the effect of various temperatures upon 20 per cent yolk sac suspensions, summarized in Table II, illustrate this thermolability. Progressive loss of activity was observed even over a few hours' time at room temperature or at 35°C. Material kept for 8 hours at room temperature and overnight at 4°C. showed almost a complete loss of activity. Storage at refrigerator temperature (4°C.) retarded this effect, although a fall in titer was observed after a 1- to 2-day period. Heating at 56°C., which destroys the infective nature of rickettsial preparations, also resulted in the complete loss of hemolytic activity.

The relation of the type of diluent employed to the stability of the hemolytic principle was investigated as a point of practical importance. Definitely higher titers were obtained when the serial dilutions of active yolk sac were made in skim milk or in 20 per cent normal yolk sac than when any other diluent was employed. Such diluents, however, have not been regularly employed owing to the impossibility of estimating end-points accurately in turbid materials. For routine use physiological saline has been adopted, since other clear materials tested, such as diluted serum or nutrient or glucose broth, yielded no better results.

High Speed Centrifugation.—Centrifugation studies were carried out with the double purpose of investigating the nature of the hemolytic principle and of removing extraneous yolk sac material, which possibly inhibited the reaction of

hemolysis and which certainly rendered more difficult the reading of hemolysis in low dilutions of yolk sac. A Pickels' angle-head centrifuge was employed to sediment the rickettsiae, and most of the work was carried out in the cold room to minimize losses due to the instability of the active material.

The results obtained (Table III) indicated that the hemolytic activity moved with the sedimentable material, the supernatant fluid being devoid of such activity. Concentration of the sediment by resuspension in a smaller volume of saline, gave rise to higher hemolytic titers. Such sedimented preparations, when resuspended to volume in saline and tested immediately, were relatively

TABLE II

Loss of Hemolytic Titer of Infected Yolk Sac Suspensions on Exposure to Various Temperatures Prior to Testing

Exposure of infected yolk sac*		Hemolytic titer† (1:∞)	
Temperature	Time	Unexposed control	Exposed
°C.			
4	1 day	64	16
4	2 days	64	32
20-25	2 hrs.	64	32
20-25	4 "	64	8
20-25	8 " §	64	2
35	2 "	32	16
35	3 "	128	32
35	4 "	32	8
56	1 hr.	32	0

* Yolk sacs were infected with either Wilmington murine or Breinl epidemic strains of rickettsiae, and were exposed in 20 per cent concentration. At the end of the exposure period, dilutions were made in saline and added to the red cell substrate.

† Titers are indicated as in Table I.

§ After exposure, stored at 4°C. overnight prior to testing.

clear and made satisfactory preparations for the demonstration of hemolytic activity despite some loss in potency during processing. The preservation of such preparations for future tests was rendered impossible, however, by their complete loss of activity upon storage, in a saline suspension, even at -70°C . (in a dry-ice chest). The latter observation is an especially marked example of the instability of the hemolytic factor. In this case, the loss of activity was apparently due to the fact that the preparations were relatively free from protective yolk sac material. Diluted rabbit serum slightly retarded the loss of potency, while skim milk proved an excellent preservative over an 8-day storage period. No practical advantage was gained, however, by the use of the latter medium since the purpose of centrifugation, i.e. to clarify the preparation and facilitate reading of the degree of hemolysis, was thereby defeated.

The Quantitative Relationship of in Vitro Hemolytic Activity to in Vivo Rickettsial Effects.—From the evidence already presented, a close relationship is suggested between the hemolytic property of suspensions of infected yolk sac and the contained, living rickettsiae. The diluents, such as normal yolk sac suspen-

TABLE III
*Centrifugation Studies on Infected Yolk Sac Suspensions**

Experiment No.	Fraction	Suspending medium†	Duration of storage‡	Hemolytic titer (1:2)	
				Un-treated control	Treated
1	Sediment Supernatant	Saline	Used immediately	32	16
			“ “	32	0
2¶	Sediment	Saline to ¼ original volume	Used immediately	128	256
3¶	Sediment	Saline to ¼ original volume	Used immediately	32	128
4**	Sediment	Saline	48 hrs.	64	0
	Sediment	25 per cent rabbit serum	Used immediately	64	32
	“	25 per cent rabbit serum	48 hrs.	64	4
5**	“	25 per cent rabbit serum	8 days	64	Undiluted
	“	Skim milk	Used immediately	64	32
	“	“ “	48 hrs.	64	32
	“	“ “	8 days	64	32

* Yolk sac suspensions were infected with Breinl epidemic or Wilmington murine strains of rickettsiae.

† Unless otherwise indicated, sediments were resuspended to original volume.

‡ Storage at -70°C . (dry-ice chest).

|| Centrifugation once at 5,000 R.P.M. for 1 hour at room temperature.

¶ Centrifugation once at 5,000 R.P.M. for 1 hour in cold room.

** Centrifugation at 5,000 R.P.M. for 1 to 1½ hours; resuspension of sediment in saline and centrifugation for 10 minutes at 1,000 R.P.M.; the supernate then centrifuged at 5,000 to 8,000 R.P.M. for 1 to 1½ hours; all done in cold room.

sions and skim milk, known to be most effective in preserving the infectivity (and hence, presumably, the viability) of rickettsial preparations, also offered the best protection for the hemolytic activity. Like the factor toxic for mice (2) the “hemolysin” could not be separated from the rickettsiae by centrifugation,

and treatment such as with 0.5 per cent formol or exposure to 56°C. for an hour, which renders rickettsiae non-infective, also destroyed the "hemolysin."

Further evidence of the relation of the "hemolysin" to the rickettsiae present was obtained by the simultaneous determination of the hemolytic titer and the titer of such other manifestations of rickettsial presence as toxicity for mice and lethality and total infectivity for cotton rats under conditions known to result in a decrease or an increase in the level of hemolytic activity. The conditions employed were exposure for 3 hours to a temperature of 35°C. (*cf.* Table II) or concentration of the active principle by resuspending the sediment obtained by high speed centrifugation in one-fourth the original volume of fluid (*cf.* Table

TABLE IV

Comparative Observations on Hemolytic Activity, Toxicity for Mice, and Lethality and Total Infectivity for Cotton Rats, of Infected Yolk Sac Pools

Rickettsial strain	Treatment of yolk sac	End-point dilutions* (1:x)			
		Hemolytic	Toxic† (for mice)	Lethal‡ (for rats)	Infective (for rats) (× 10 ⁶)
Wilmington murine	Fresh	128	22.3	42.5	1.3
	3 hrs. at 35°C.	32	5.3	12.9	0.45
	4 × concentrated	256	77.6	128.0	10.0
Breinl epidemic	Fresh	128	56.1	306.0	10.8
	3 hrs. at 35°C.	32	13.1	11.3	5.4
	4 × concentrated	512	181.0	512.0	22.0

* Starting material (20 per cent suspensions of yolk sac) considered as undiluted. Inoculum for rats and mice, 0.25 ml. volume. End-points other than hemolytic calculated by 50 per cent method (9).

† Based on 8 mice per twofold dilution.

‡ Based on 5 (Breinl) or 10 (Wilmington) rats per twofold dilution.

|| Based on 8 rats per fourfold dilution.

III). The results of two such experiments, one with a yolk sac pool infected with the Wilmington strain of murine typhus rickettsia and the other with a pool infected with the Breinl strain of epidemic typhus rickettsia are presented in Table IV and are typical of those obtained. With due regard for the limits of error in the various titration methods employed, the parallelism observed between the decline of the several activities measured (time-temperature effect) and their respective increases (as the result of concentration by centrifugation) is striking.

Observations on the Mechanism of the Hemolytic Reaction

It has been deduced that the hemolytic principle is intimately associated with the actual rickettsial bodies. Since these bodies are visible with the aid of a

microscope, it seemed possible that examination of Giemsa-stained smear preparations of hemolyzing yolk sac-substrate mixtures taken after various intervals of incubation would reveal something of the mechanism involved. Except in the 0-hour preparation, in which some indication of the attachment of rickettsiae to the cell margins was seen, the examination of a series of such smears revealed no relation whatsoever between cells and rickettsiae. The principal finding was that in smears taken after progressively longer intervals of incubation the red cells became less abundant and presented evidence of lytic changes with pale, indistinct margins. Quantitative evidence of the progressive reduction in the number of cells present was obtained by several series of counts made by the usual clinical laboratory technique at various intervals after combining yolk sac and substrate. These counts are shown in Table V. In spite of irregu-

TABLE V

Red Cell Counts on Hemolyzing Yolk Sac-Substrate Mixtures after Different Intervals of Incubation

Experiment No.	Rickettsial strain	Dilution of yolk sac	Red cell count (millions) at intervals of incubation (35°C.)						
			0	5-15 min.	30 min.	1 hr.	2 hrs.	4-5 hrs.	18 hrs.
1	Epidemic	1:2	0.73	0.60	0.62	0.62	0.25	0.20	0.15
2	Murine	1:2	2.09	2.01	1.50	—	1.22	0.65	—
		1:4	—	2.33	1.51	—	1.25	0.90	—
		Blank	—	—	—	—	—	1.90	—
3	Murine	1:2	—	—	1.50	—	0.84	0.70	—
		1:4	—	—	1.46	—	1.09	1.06	—
		Blank	1.37	—	—	—	—	1.66	—

larities, it is evident that the rate of the decline in count was related to the concentration of yolk sac employed, but that even with the most concentrated yolk sac preparation (1:2 dilution) a 50 per cent reduction in count did not occur much before the 4th or 5th hour of incubation.

Although direct microscopic examination failed to reveal a clear relation between cells and rickettsiae, it seemed of interest to determine whether cells once exposed to infected yolk sac would continue to undergo hemolysis when removed from the hemolytic system and, conversely, whether such removal of the cells would have a significant effect upon the hemolytic potency of the remaining portion of the system. In these studies, substrate and serial dilutions of yolk sac infected with rickettsiae of a murine typhus strain were allowed to remain in contact for 15 minutes in a water bath adjusted to 37°C. or to 5°C., after which the cell component and supernatant (yolk sac plus serum and saline of the substrate) were separated by brief, light centrifugation. The washed or un-

washed red cells were resuspended in new supernatant fluid composed of appropriate dilutions of normal yolk sac plus rabbit serum and saline to replace the fluid portion of the substrate, while fresh packed cells were added to the original supernatant. Both new series with the indicated control series were then incubated for 18 hours at 35°C. before reading.

TABLE VI
Studies of Possible Adsorption of Hemolytic Activity by Red Cells

Temperature of preliminary incubation °C.	Fraction*	Final hemolytic titer† (1:2)
35	Control mixture	256
	Supernatant	256
	Red cells	64
5	Control mixture	256
	Supernatant	256
	Red cells	64
35	Control mixture	128
	Supernatant	128
	Red cells	32
	Washed once§	8

* Equal volumes (1.0 ml.) of substrate (25 per cent cells) and yolk sac dilutions mixed; separated after 15 minutes' incubation by centrifugation at 1,000 r.p.m. for 5 minutes; supernatant tested with fresh cells; cells tested with fresh supernatant prepared with normal yolk sac.

† Dilutions showing a trace of hemolysis after 18 hours' incubation at 35°C.

§ Cells washed with 3.0 ml. (12 volumes) of saline and recentrifuged.

The results of the studies (Table VI) fail to indicate any marked tendency for the hemolytic principle to be adsorbed onto the red cells. In no case was the titer of the supernatant fluid, after a preliminary exposure to red cells, lower than that of the control series, and in all cases the titer observed with exposed red cells after replacement of infected supernatant material with normal material, was demonstrably lower than that of the control. This is in accord with our failure to observe microscopically any adherence of rickettsiae to the red cells and with the evidence that the hemolytic principle is inseparable from the

living rickettsial organisms. Some hemolysis of exposed red cells did occur, however, after their removal from the infected supernatant fluid, and, indeed, even after a single washing in saline. Such residual hemolysis, after washing, was found only in the case of those cells which had been exposed to high concentrations of rickettsiae (sufficient to result in 4+ hemolysis with control and supernatant material). It is probable that the explanation lies in the nature of the hemolytic phenomenon rather than in any true adsorption.

It has been shown previously that the hemolytic process is a slowly progressive one and is apparently dependent upon the rather gradual infliction of damage on the red cells. What the nature of the effect upon the cell may be is not known, although in one experiment, in which somewhat hypotonic saline was employed, the cells did not exhibit increased fragility. Since the hemolytic principle undergoes destruction at temperatures favoring the demonstration of its presence, the degree of hemolysis observed must be the resultant of two opposing factors: (1) the rate of red cell injury and (2) the rate of "hemolysin" destruction. The rate of red cell injury must be determined, in part, by the concentration of active principle (apparently viable rickettsiae), so that, whereas short exposure to high concentrations will result in some damage to the red cells, prolonged exposure to low concentrations, because of the simultaneous inactivation of "hemolysin," may result in little hemolysis. Accordingly, washing of red cells exposed to high concentrations of rickettsiae will not prevent those cells already extensively damaged from subsequently undergoing lysis, while simple separation of cells from the original supernatant fluid by centrifugation will, in addition, leave behind some active material to carry further the damage initiated during the preliminary exposure period. It can be seen that it is impossible to quantitate the results obtained, even approximately, in view of the inadequacy of our knowledge concerning the factors governing the rates of reaction. The fact that some but not all the erythrocytes undergo hemolysis with any preparation is probably due to variations in susceptibility among the cell population and to loss of activity of the preparation occurring before the more resistant cells have been sufficiently affected to undergo lysis.

The Inhibition of Hemolysis with Immune Serum

As has been indicated, early studies of the hemolytic principle showed that its activity could be inhibited by homologous antiserum. In order to investigate this inhibition more fully the optimal conditions for its demonstration had to be determined. A detailed presentation of this preliminary phase of the work will be omitted in favor of data showing the application of the technique to serological studies. In summary, the best results were obtained when equal volumes (0.2 ml.) of infected yolk sac suspensions and dilutions of immune serum, made with saline, were mixed and allowed to remain in contact for 30

minutes at room temperature prior to the addition of the red cell substrate (0.5 ml.). The final mixture was then incubated for 18 hours at 35°C. Simultaneously, control tubes were prepared which contained saline instead of the serum dilutions. The use of too great a concentration of the hemolytic factor resulted in specific serum inhibition titers which were unduly low. Exceedingly weak concentrations, on the other hand, operated to diminish the specificity of the test by permitting the demonstration of inhibitory activity with normal sera. The best compromise was obtained when dilutions of yolk sac giving 2+ or 1 to 2+ hemolysis were employed against serial dilutions of immune serum. It may be recalled at this point that sera of species other than the rabbit occasionally cause hemolysis of rabbit cells and that this generally can be avoided by heating the sera at 56°C. for $\frac{1}{2}$ to 1 hour prior to their incorporation in the test. The very occasional persistence of this activity after heating makes it advisable to use control mixtures of serum and cells to indicate false reactions.

Some suggestion was obtained that the addition of fresh normal guinea pig serum (unheated) somewhat enhanced the antisera titers. The possible rôle of complement in the process was thus indicated, but the frequent hemolytic action of unheated guinea pig serum upon rabbit cells made the addition inadvisable as a routine procedure.

Because of an unexplained difficulty in the production of satisfactory yolk sac pools of strains of murine typhus rickettsiae, most of our experience with the serum inhibition technique has been with the rickettsiae of epidemic typhus. A few satisfactory tests have been made with murine typhus rickettsiae, however, and some of the results obtained with both types are shown in Table VII. The data presented indicate the specificity of the test when applied to cotton rat antisera obtained against either rickettsial strain. Complete inhibition was shown with homologous antisera in dilutions of 1:16 to 1:32 and partial inhibition in dilutions of 1:128 or higher. In contrast, complete inhibition was rarely observed with either normal or heterologous sera, and the degree of partial inhibition was not marked. With rickettsiae of epidemic typhus and sera of humans convalescent from this disease, titers comparable to those seen with animal sera were obtained, and the normal human sera tested showed little non-specific inhibition. High titers were likewise observed with the convalescent human sera against a "hemolysin" of murine typhus origin, however, so that the strain specificity for human studies remains somewhat open to question. Owing to the lack of available potent preparations of murine typhus rickettsiae and of human antiserum against these rickettsiae, this question of strain specificity could not be investigated more fully.

In Table VIII, results of serum inhibition tests with various human and rabbit sera against yolk sac infected with the rickettsiae of epidemic typhus are compared with the results of complement fixation tests, and when available, toxin neutralization tests obtained with the same sera. The use of complete

inhibition of hemolysis as the criterion of activity results in a test of low sensitivity in comparison with other serological methods. However, with the acceptance of partial inhibition as the index, the sensitivity of the technique is

TABLE VII
Antihemolytic Titers of Human and Animal Sera

Rickettsial strain of hemolysin*	Sera†	Antihemolytic titers (1:±)	
		Complete‡	Partial§
Murine	Murine (cotton rat)	32	>128
	Epidemic (" ")	Undiluted	16
	Normal (" ")	0	2
Epidemic	Epidemic (cotton rat)	16	128
	Murine (" ")	0	16
	Normal (" ")	0	0
Epidemic	Epidemic (human No. 1)	32	>256
	" (" " 2)	8	16
	" (" " 3)	16	128
	" (" " 4)	32	>256
	" (" " 5)	32	128
	" (" " 6)	8	32
	" (cotton rat)	16	>64
	Normal (" ")	Undiluted	4
	" (human)	0	8
	Epidemic (human No. 2)	64	128
Murine	" (" " 4)	64	128
	" (" " 5)	>256	>256
	" (" " 6)	8	16
	" (cotton rat)	2	32
	Murine (" ")	32	128
	Normal (" ")	0	0

* Hemolysin preparations were dilutions of yolk sac suspensions giving a 2 + hemolysis in the absence of test sera.

† The cotton rat antisera were pools obtained from animals surviving infection with the indicated strain and bled 2 to 3 weeks after inoculation. The human antisera were obtained in 1941 from Spanish prisoners convalescent from typhus fever and were stored under sterile conditions at 4°C. until used.

‡ Highest dilution of serum showing complete inhibition of all hemolytic activity.

§ Highest dilution of serum showing significant reduction in degree of hemolysis as compared with control containing saline instead of serum.

comparable to that of the method of complement fixation employed (10), except in the case of the sera from vaccinated rabbits, in which, particularly as related to adjuvant vaccine (11), a tremendous discrepancy occurred. In the rabbit

TABLE VIII

Comparison of Hemolysin Inhibition, Complement Fixation, and Toxin Neutralization by Immune Sera (Epidemic Typhus Antigens Only)

Serum				End-point dilution (1:x) of serum as measured by			
Species	Individual	History	Time interval	Inhibition of hemolysis*		Complement fixation†	Toxin‡ neutralization
				Complete*	Partial*		
Human	D. C.	Post-typhus vaccination	2 wks.	1	64	50	—
		Laboratory infection (murine)	21 days	1	32	128	—
	J. F.	Laboratory infection (vaccinated)	21 days	1	64	128	—
			31 "	1	64	128	—
	J. S.	Post-typhus vaccination	2 wks.	1	32	24	—
		Laboratory infection (epidemic)	7 mos.	8	128	128	—
	O. P.	Laboratory infection type unknown (vaccinated)	15 days	8	128	512	—
			40 "	16	256	512	—
	C. A.	Post-typhus vaccination	2 wks.	4	64	16	—
		Laboratory infection (epidemic)	14 days	32	>512	1500	—
Rabbit	No. 21	Adjuvant vaccine (epidemic)	8 wks.	2	>128	1240	570
			13 "	4	>128	840	900
			20 "	8	64	400	250
			35 "	1	32	190	250
	No. 37	Saline vaccine (epidemic)	3 wks.	1	2	24	3
			8 "	0	1	21	0
			13 "	0	0	15	0
			35 "	0	0	15	0

* See preceding table.

† 50 per cent hemolysis end-point (10) with 2 units of antigen and 2 full units of complement.

‡ Calculated by the 50 per cent end-point method (9); measured against ± 2.5 I.D.₅₀ of toxic yolk sac.

|| Vaccine incorporated in water-in-oil emulsion (11).

series, also, the toxin neutralization titers far surpassed the inhibition titers based on either criterion. The number of tests performed up to the present

time has not been sufficient to indicate the true significance of the phenomenon of inhibition.

*Note on the Mechanism of the Toxicity of Rickettsia-Infected
Yolk Sac Suspensions for Mice*

The possible relation of the phenomenon of hemolysis to that of the toxicity of rickettsia-infected yolk sac was suggested naturally by the high degree of parallelism observed in the respective properties of the "hemolysin" and the "toxin" and also by the fact that sera from mice bled during the acute phase of infection with murine typhus rickettsiae (in a study of the development of immunity) consistently evidenced a high content of dissolved hemoglobin. In spite of the fact that mouse red blood cells, *in vitro*, appeared to resist hemolysis, it seemed possible that different conditions *in vivo* might permit hemolysis to occur.

Mice were inoculated intravenously with two or three dilutions of yolk sac richly infected with rickettsiae, with the intention of bleeding the animals at intervals and following, by serial determinations, their blood hematocrit readings and the hemoglobin content of their plasma. The results were unexpected in that the principal toxic effect appeared to be a marked reduction in blood volume. To begin with, it proved nearly impossible to obtain more than 0.1 to 0.2 ml. of blood from the heart of a mouse with signs of toxicity, whereas volumes of from 0.5 to 1.0 ml. were ordinarily taken from normal mice. This difficulty was shortly explained when the cell volumes had been obtained on pooled specimens of heparinized blood. Whereas the cell volumes from normal mice were from 4.0 to 4.5, readings of 5.5 to 7.2 were obtained in several experiments with blood drawn from mice near death following toxic inocula. That the mechanism of toxicity is due to a seriously altered vascular permeability is indicated by the fact that, in spite of the increased cell concentration, the specific gravity of the plasma remained essentially unchanged, ranging from 1.018 to 1.022 in normal mice and in those with toxic signs alike, as determined by the copper sulfate method (12).

Death of the mice following toxic inocula thus was apparently a shock phenomenon. Subsequent experiments were made with animals given additional fluid (2.0 ml. of saline intraperitoneally) or such antihistamine drugs as adrenalin or pyribenzamine. None of these measures affected either the survival of the mice or the hematocrit readings obtained. However, mice inoculated with rickettsia-infected yolk sac mixed with homologous antiserum not only survived but showed no blood changes.

From these experiments it appears that toxicity of rickettsia-infected yolk sac for mice is dependent upon a factor which gravely alters vascular permeability. Whether or not it is this same factor which causes *in vitro* hemolysis

of the blood of other animal species by altering the permeability of the membranes of red cells, is still undetermined.

DISCUSSION AND SUMMARY

Suspensions of yolk sac infected with rickettsiae of murine or of epidemic typhus, and indeed suspensions of liver or peritoneal washings from cotton rats infected with these organisms, when the suspensions contain a sufficient concentration of living rickettsiae, possess the capacity to hemolyze, *in vitro*, red cells of rabbit or sheep origin. Under the same conditions such material did not cause the hemolysis of cells from mice, cotton rats, or guinea pigs. Yolk sac and tissue suspensions of *R. orientalis* (Karp, Raub, and Gilliam strains) failed to hemolyze rabbit cells.

The hemolytic principle was destroyed by formol (0.5 per cent) and by heating to 56°C. for 1 hour. The close association of the "hemolysin" with the rickettsiae was indicated by their parallel movements on centrifugation and by the close parallel demonstrated between the effects produced on hemolytic activity and on such manifestations of the presence of living rickettsiae as toxicity for mice and lethality or total infectivity for cotton rats when infected yolk sac suspensions were exposed to elevated temperature or were concentrated by high-speed centrifugation.

The mechanism of hemolysis remains ill defined. It progresses slowly, but never to completion (*i.e.* hemolysis of all the cells in the substrate) even when the number of such cells is small. This may be because hemolysis progresses best at temperatures ($\pm 35^{\circ}\text{C}.$) which are unfavorable to the hemolytic principle and because erythrocytes vary in their susceptibility to the injurious agent. The concentration of red cells also may be a determining factor, either by affecting the rate at which the reaction proceeds or by aiding in the preservation of hemolytic activity. Although it was impossible to separate the "hemolysin" from the rickettsiae, examination of smear preparations of hemolyzing mixtures showed no consistent contact relation between cells and rickettsiae. Adsorption experiments revealed that, while removal of the cells from the hemolytic system did not reduce the hemolyzing capacity of the system, the cells carried away a significant tendency to hemolyze. It is suggested that the rate at which injury is inflicted on the red cells is dependent on the concentration of "hemolysin" present and that the process may proceed in the relative absence of rickettsiae once sufficient damage has been inflicted.

The toxicity of richly infected yolk sac suspensions for intravenously inoculated mice was found to be related, not to *in vivo* hemolysis, but apparently to a serious alteration in vascular permeability which caused a marked reduction in blood volume. This was evidenced by an increased concentration of red cells but not of plasma proteins. It is possible, but has not been demonstrated,

that the factor responsible for altering vascular permeability of mice may be identical with that which causes lysis of rabbit and sheep cells.

Two applications of the phenomenon of *in vitro* hemolysis are indicated. Following the technique described in the text for the optimum demonstration of hemolysis, the determination of the degree of hemolytic activity may serve as a rapid, roughly quantitative *in vitro* method for assessing the infectivity of a newly made rickettsial preparation. The phenomenon also is the basis for an additional, and potentially useful *in vitro* serologic technique which differs from other techniques in that it depends on a property of living rickettsiae. It has been shown that homologous antisera are capable of inhibiting hemolysis, and optimum conditions for demonstrating this inhibitory effect are described.

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IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

VII. CHEMICAL CHANGES ASSOCIATED WITH DESTRUCTION OF BLOOD GROUP ACTIVITY AND ENHANCEMENT OF THE TYPE XIV CROSS-REACTIVITY BY PARTIAL HYDROLYSIS OF HOG AND HUMAN BLOOD GROUP A, B, AND O SUBSTANCES*

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In a previous study (1), it was reported that preparations of purified blood group A substances from individual hog stomachs varied in their capacity to precipitate with Type XIV antipneumococcal horse sera. Nevertheless these substances had the same blood group A activity (2, 3) as determined by quantitative immunochemical methods (*cf.* references 4-7). Blood group O substances from individual hog stomachs as well as preparations of human blood group substances also showed similar variations in their cross-reactivity with Type XIV antiserum. From these observations, it seemed that the two activities might be associated with different portions of the complex molecule and it was predicted that it might be possible to alter one of the two activities without influencing the other (1). The effect of heating the hog blood group A and O substances at various pH on cross-reactivity with Type XIV antiserum was therefore studied as had been done previously for the blood group A activity (2). It was found that exposure to 100°C. in dilute hydrochloric acid at a pH of 1.5-2.0 for 1 to 2 hours resulted in almost complete destruction of blood group A and O activity but strikingly increased the reactivity with Type XIV antiserum. Similar results were obtained on heating A and B substances from human saliva. Study of the chemical changes associated with this procedure showed that about 60 to 80 per cent of the fucose of the hog A and O substances (8) and some of the amino acid nitrogen together with small amounts of free galactose and glucosamine became dialyzable. In addition some dialyzable polysaccharide was also found. From the non-dialyzable portion, products were isolated by alcohol precipitation which had an enhanced reactivity with Type XIV antiserum and had a lower methylpentose content than the original material. These data, together with the findings of Bray, Henry,

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and Stacey (9) that the fucose was present as end groups, strongly indicate that both the blood group A and O substances are made up of long chains of *N*-acetylglucosamine and galactose residues with the fucose residues projecting outward as end groups at various undetermined points along the chain. These fucose residues reduce the reactivity of the blood group substances with Type XIV antiserum and their removal by heating at pH 1.5-2.0 results in the observed increased reactivity with Type XIV antibody. The methylpentose in blood group A substance from human saliva was identified as fucose by paper chromatography.

EXPERIMENTAL

The hog and human blood group substances employed were those described in earlier studies (3, 8, 10). Type XIV antipneumococcal horse serum H635 (*cf.* reference 1) supplied by Dr. Harold W. Lyall and Miss Jessie L. Hendry of the New York State Department of Health contained 0.87 mg. anti-SXIV N¹ per ml.

The stability of the Type XIV activity of blood group A and O substances after heating at various pH was studied as follows: To 1.50 ml. samples of a saline solution containing 2.00 mg. of blood group substance per ml., 1.50 ml. of solutions of appropriate pH (*cf.* reference 2) from about 1.5 to 10.8 were added; the pH was determined after mixing. The tubes were sealed and heated in a boiling water bath for 2 hours. After heating, 2.50 ml. aliquots were removed, neutralized with acid or alkali if necessary, and diluted to 5.00 ml. in a volumetric flask with saline to give a solution containing 500 μ g./ml. The cross-reactivity of each heated solution with Type XIV antiserum was determined at 0° by adding volumes containing 50, 100, 150, 250, and 500 μ g. of blood group substance to 0.5 ml. portions of antiserum in a total volume of 2.0 ml. After 1 week in the refrigerator the precipitates were centrifuged off, washed twice in the cold with saline, and analyzed for nitrogen (11) by the Markham micro-Kjeldahl method (19). The blood group A activity was determined by comparing the minimum quantity of each heated solution of blood group A substance required for inhibition of hemagglutination of human A erythrocytes by human anti-A (2) with that of an unheated saline solution of the same preparation of A substance. Blood group O activity was assayed in a similar manner using O erythrocytes and a goat anti-Shiga serum (12) generously supplied by Dr. A. F. Coca of Lederle Laboratories. The serum contained anti-O and was used after absorption with A₁B cells.

RESULTS

Table I shows the effect of exposure to various pH at 100°C. for 2 hours on the Type XIV and blood group activity of solutions of two preparations of hog blood group A substance, on three samples of hog blood group O substance, and on one A preparation, hog 15 (S), which contained some O substance. It is apparent that, as compared with the corresponding unheated sample, the heated materials showed greatly enhanced capacity to precipitate Type XIV antibody after heating at 100° for 2 hours at pH ranging from 1.49 to 1.9 and that the blood group activity of these substances had been very extensively or almost completely destroyed. The resulting increase in the cross-reactivity

¹ SXIV the type-specific polysaccharide of Type XIV pneumococcus.

appeared to be greater for the blood group O substances than for the A substances. As a control, a sample of SXIV heated under similar conditions showed a slight decrease in reactivity with Type XIV antiserum.

TABLE I

Effect of pH on Cross-Reaction of Blood Group Substances with Type XIV Antipneumococcal Serum. Tubes Heated 2 Hours at 37° at Indicated pH, Neutralized

Total N precipitated from 0.5 ml. antiserum H635

Amount of substance added μg.	pH										pH				
	1.52	1.71	2.79	4.69	6.71	7.40	8.8	9.15	9.49	Unheated	1.9	4.72	7.5		Unheated
	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
Hog 16 (A)											Hog 13 (O)				
50	24	29	6	10	9	2	0	2	1	12	26	15	6		16
100	46	43	10	16	14		2	0	2	18	45	26	12		27
150	54	59	14	23	18	4	1	1	1	24	57	36	18		39
250	61	75	27	24	22	9	3	2	3	30	69	43	25		43
500	70	95	46	38	32	14	0	2	5	39	97	64	36		64
Agglutination-inhibiting power*	>10	>50	1	1	1	2	10	50	50	1	>25	>25	10		2
Hog 15 (AO)											Hog 25 (O)				
	1.49	1.51	2.90	4.75	6.91	7.75	8.8	9.62	10.05	Unheated	1.61	4.70	7.71	9.05	Unheated
50	24	20	17	7	6	7	2	0	1	8	50	12	8	2	11
100	43	29	26	12	9	9	2	1	1	13	69	18	11	2	14
150	54	43	34	14	14	16	4	0	1	17	86	21	11	4	18
250	67	57	47	22	21	24	3	2	3	23	107	25	14	4	20
500	84		74	39	35	32	3	4	1	35	128	37	20	7	27
Agglutination-inhibiting power	>50		1.5	1	1	2	50	50	50	1	>50	1-2	5-10		1
Hog 10 (A)											Hog 27 (O)				
	1.59		2.95	4.59	6.55	7.50				Unheated	1.52	4.71	7.71	8.9	Unheated
50	14		5	7	5	3				13	35	28	23	5	29
100	24		9	6	6	5				8	61	46	32	5	44
150	30		12	8	7	4				13	76	61	40	11	51
250	46		14	15	10	2				16	102	81	54	14	64
500	64		28	17	14	5				19	139	99	69	23	77
Agglutination-inhibiting power	>50		0.2	0.1	0.1	0.1				0.1	>50	10	25		5

* Minimum quantity of A or O substances completely inhibiting hemagglutination of A cells by anti-A or of O cells by anti-O.

† Highest dilution tested showing no hemolysis.

With the three A products, heating at pH 2.79-2.95 seemed to result in a slight increase in cross-reactivity without significant loss of blood group A activity as measured by hemagglutination (*cf.* reference 2) but similar treatment at pH from 4.59-6.91 did not significantly affect either the cross-reaction or the

blood group A activity. At pH 7.4–7.5, 100°C. for similar periods produced some destruction of both cross-reacting and blood group activity in two instances, but with the hog 15 preparation, no significant change occurred; at pH 8.8 or above extensive loss in both activities resulted.

With the O substances heating at pH 7.5–7.71 resulted in a fivefold decrease in blood group O activity and in some diminution of cross-reacting potency. At pH 8.9–9.0 marked diminution of cross-reactivity occurred in the two instances studied. At pH 4.7 some decrease in blood group activity with no change or but a slight increase in cross-reactivity was noted.

Table II shows that the increase in cross-reactivity produced at pH 1.6 was greatest after 1 hour at 100° and decreased with further heating.

TABLE II

Effect of Time of Heating at pH 1.6 on Cross-Reaction with Type XIV Antipneumococcal Serum

Amount blood group substance* added	Total N precipitated from 0.5 ml. antiserum H635			
	Time of heating			
	1 hr.	2 hrs.	4 hrs.	6 hrs.
μg.	μg.	μg.	μg.	μg.
50	43	36	17	12
100	66	52	27	14
150	87	67	36	19
250	107	87	46	21
500	146	118	66	43

* Hog 13 O substance used.

Similar increases in cross-reactivity were obtained on heating three samples of A substance and one of B substance from human saliva (10) (Table III).

Chemical Changes Associated with Heating at pH 1.5–1.8.—

Chemical changes associated with the increase in reactivity with Type XIV antiserum and the destruction of blood group activity which resulted from heating at pH 1.5–1.9 were investigated using the hog 15 substance which was predominantly blood group A substance (8) and the hog 29 material a preparation of blood group O substance.

Solutions containing about 5 mg. per ml. of the purified substances from hogs 15 and 29 were prepared and acidified to pH 1.5 and 1.8 respectively; total nitrogen, glucosamine, and reducing sugar before and after hydrolysis, methylpentose (13), cross-reactivity, blood group A or O activity, and specific optical rotation were measured. The solutions were then heated in sealed tubes in a boiling water bath for 2 hours and 1 hour respectively and the same analytical data obtained (Table IV). Measured volumes of the heated solutions (containing 260 mg. of hog 15 substance and 265 mg. of hog 29 substance) were then dialyzed against four and five 75 ml. portions of distilled water. The non-dialyzable portions were removed

from the cellophane sacs, 2 gm. of sodium acetate was added to each, and the material was fractionally precipitated with ethanol. Most of the material was precipitable by 2 volumes

TABLE III

Effect of Heating at 100° for 2 Hours at Acid pH on Cross-Reactivity of Human Blood Group Substances

Amount substance added	Total nitrogen precipitated from 0.5 ml. H635	
	Heated 2 hrs.	Unheated
$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$
A substance: W.H. ₂ digest of water-insoluble, phenol-insoluble pH 2.18		
50	30	28
100	46	43
150	61	53
250	81	69
500	121	90
A substance: B.K. phenol-insoluble pH 1.99		
50	49	33
100	67	53
150	84	67
250	114	89
500	156	118
A substance: W.H. ₂ digest of water-insoluble 10 per cent precipitate pH 1.72		
50	47	25
100	74	45
150	96	50
250	118	66
500	163	83
B substance: S.E. phenol-insoluble pH 1.90		
50	41	20
100	64	31
150	81	36
250	111	42
500	144	56

of ethanol (P1) and a small additional portion was obtained between 2 and 5 volumes (P2). In the case of hog 15 only an additional 3 mg. of substance was obtained by adding an equal volume of acetone to the ethanol supernatant. The yields, analytical properties, blood group, and cross-reactivity of these fractions are shown in Table IV.

Aliquots of the dialysates were analyzed and the remainder of the dialysates was concentrated *in vacuo* under CO₂ to a volume of 2.5 ml. and chromatographed on Whatman number 1 filter paper (14) using in separate experiments butanol-ethanol-water (40:10:50) and collidine-water as solvents as described by Partridge (15).

Qualitative identification of the constituents was made on the same paper by simultaneous comparison of the R_F values of the constituents of the concentrated hydrolysate with those of fucose, galactose, and glucosamine. With both solvents, fucose and galactose were found in the concentrated dialysates of both the hog 15 and hog 29 substances when chromatograms were developed by heating with alkaline copper or with ammoniacal silver. With collidine, glucosamine could also be identified but only in the hog 15 dialysate and in very small amounts.

TABLE IV

Properties of Unheated and Heated Hog Blood Group Substances and of the Non-Dialyzable Fractions Obtained after Heating at pH 1.5 and 1.8

	Ash as Na	Total N	Re- duc- ing sugar		Gluc- osa- mine		Re- duc- ing sugar		Gluc- osa- mine		Methylpentose	[α] _D	Acetyl	Mini- mum hema- agglu- tination inhib- iting dose	Total N precipitated from 0.5 ml. type XIV serum by					Amount of substance
			On hy- drolysis*		Unhy- drolyzed		50 μg. substance	100 μg. substance	150 μg. substance	250 μg. substance					500 μg. substance					
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	μg.	μg.	μg.	μg.	μg.	μg.	mg.	
Hog 15		5.5	59	29	11	1.2	9.2	+10°					0.5	8	13	17	23	35	260†	
Hog 15 heated			57	27	22	3.2		+23°					>150	29	45	60	82	130		
P1	0.2	6.0	56	31	12	1.7	1.7	+30°	11.2				>100	39	56	68	81	100	120	
P2		6.8	59	33		2.0	1.5	+25°					>100	27	40	50	68	92	12‡	
Hog 29		6.0	58	27	10	1.8	9.5	- 5°					1	21	32	41	57	88	265‡	
Hog 29 heated			60	29	17	2.4		+ 7°					>100	66	104	131	161	200		
P1	0.2	5.8	56	29	15	1.2	3.7	+10°	10.3				>150	73	105	128	148	173	213	
P2			48	26	16	1.2	3.0						>150	42	61	76	96	120	8	

* With 2 N HCl for 2 hours at 100°C.

† Amount initially used, other figures in column indicate yield.

‡ An additional 3 mg. of material containing 2.1 per cent methylpentose and 27 per cent glucosamine and which also reacted with Type XIV antiserum was isolated from the P2 supernatant by addition of an equal volume of acetone.

However, in both solvents a definite spot remained at the starting point with the two concentrated dialysates; this was designated as the slow spot. In addition, when chromatograms were developed with ammoniacal silver a very fast moving spot was noted with each solvent in both the hog 15 and hog 29 dialysates. The R_F values of this spot ranged from about 0.40 to 0.44 in butanol and 0.61 to 0.64 in collidine. No such spot was obtained when chromatograms were developed with alkaline copper. This spot was shown to be due to glycerol, and control experiments showed it to come from the dialyzing membranes and not from the blood group substances. Its identification as glycerol was made by measuring its R_F values and preparing the triphenylurethane on material isolated from the fast spot.

To obtain more precise information about the composition of these concentrated dialysates, the technic of paper chromatography was adapted to quantitative estimation of the various sugars, modified from qualitative and

quantitative procedures for sugars and amino acids used by other workers (14-18).

Sheets of Whatman number 1 filter paper measuring 19×46 cm. were used. A pencil line, perpendicular to the length was drawn 10 cm. from one end of the paper and exactly 0.10 ml. of the concentrated dialysate to be examined was distributed from a calibrated 0.10 ml. pipette as a series of small spots along this line allowing margins of about 2 cm. at each end. In each margin, a small additional drop of the dialysate was placed. When the spots were dry the end of the paper was placed in the trough containing the desired solvent and the chromatograms developed at room temperature for about 20 hours when butanol was used and 24 to 26 hours with collidine. Duplicate papers were chromatographed simultaneously in each solvent. The papers were then removed, the position of the solvent boundary marked, and the papers dried at room temperature in a chemical hood in a strong current of air. The marginal strips were cut off and the spots on one strip were developed by spraying with alkaline copper and those on the other with ammoniacal silver and heating in an oven at 110°C . Using these developed margins as a guide, strips of measured width were cut at right angles to the marginal strips from the main pieces of paper so as to include each spot as completely as possible. When inadequate separation of spots was obtained a single strip containing two spots was cut. In this manner, strips containing the fast component, the fucose, the galactose plus glucosamine, and the slow component were obtained. Blank strips of measured width were cut from areas where no spots were evident but which had become wet with solvent. The strips containing the various constituents were marked with pencil and residual solvent was removed under vacuum in a desiccator containing vessels of concentrated sulfuric acid, phosphorus pentoxide, and pellets of sodium hydroxide; the desiccator was placed in an incubator at 37°C . for from 2 to 4 days. This procedure was found necessary to reduce blank values to a minimum in the subsequent analytical procedures; the longer drying period is preferable for chromatograms developed with collidine. The papers were then removed and strips containing a given component from the duplicate papers were placed in a test tube containing an analytically measured volume of distilled water (usually 10 or 15 ml.), stoppered with rubber stoppers, and allowed to stand overnight at room temperature to extract the various constituents from the paper; the blank strips of paper were extracted similarly. The solvent was decanted from the strips of filter paper and as large a quantity as possible recovered by pressing the paper with a stirring rod. The extracts were then centrifuged at room temperature to remove lint. Aliquot portions of the extracts corresponding to the various spots and blanks were analyzed for reducing sugar and glucosamine, both before and after hydrolysis for 2 hours with 2 N HCl, for methylpentose by the method of Dische and Shettles (13), and in the case of papers developed with butanol-ethanol-water for nitrogen by the Markham (19) micro-Kjeldahl method. Values obtained were corrected for the blank by calculating the blank according to the width of paper taken for each individual strip. The amounts found in the 0.2 ml. of each concentrated dialysate placed on the two papers and the total amount of each constituent in the entire dialysate were calculated. The results obtained are given in Table V. Since blank values were appreciable in all instances and since differences were multiplied by very large factors, low values are not considered significant; such values are given in parentheses in Table V. It is also possible that some small quantities of a given constituent were accidentally included in the adjacent strip by inadequate separation in the chromatograms or by tailing (14, 16). It will be noted from Table V, that recoveries of total reducing sugar, nitrogen, and methylpentose are in close agreement with those found in the total dialysate; recoveries of total glucosamine were somewhat poorer. No values are included for reducing sugar and glucosamine after hydrolysis or for glucosamine before hydrolysis when collidine was used as solvent since reproducible values could not be obtained under these conditions with this solvent.

TABLE V—*Continued*

	Paper chromatography of hog 29 dialysate									
	Total dialysate hog 29	30.6	15.3	7.1	1.2	17.9	2.2	1.6		
Slow moving		11.6	1.2	0.8	3.1	(0.6)	(0.2)	0.87	0.00	
Galactose		2.0	2.2	0.6	0.9	(0.1)	(0.2)	0.0	0.09	0.08
Glucosamine		14.4	11.31	11.15	1.1	(0.7)	14.2	0.0	0.22	0.22
Fucose		(0.0)	(0.0)	(0.2)	(0.0)	(0.1)	(0.1)	(0.7)	0.40	0.41
Fast moving										
Total in dialysate calculated from paper		28.0	11.7	12.7	5.1	15.1	14.9	2.3		0.00

Values in parentheses may not be significant due to low value found variations in blanks and large conversion factors.

* Average of nitrogen determinations on three independent sets of paper chromatograms.

† To convert reducing sugar to fucose multiply $\times 3/2$ giving 22.0 and 23.0 mg. for hog 15 and 16.6 mg. for hog 29.

‡ In repeated runs, values of 1.1 and 1.4 mg. of methylpentose found; probably the differences are due to variations in the position at which the papers were cut to separate the components. Similar variations were found with the galactose-glucosamine and the slow spot with relation to the separation of glucosamine and reducing sugar.

§ Spots demonstrable only by spraying with ammoniacal silver and not with alkaline copper. The glycerol was shown to come from the dialysis sac.

* Average of two independent sets of nitrogen determinations.

In the case of the hog 15 substance, heating at 100°C. for 2 hours at pH 1.5 resulted in an increase in the unhydrolyzed reducing sugar from 11 to 22 per cent and in the glucosamine from 1.2 to 3.2 per cent. For the 260 mg. of substance used, this amounted to 28.6 and 5.1 mg. respectively. This was accompanied by an increase in optical rotation from $+10^\circ$ to $+23^\circ$, essentially complete loss of blood group A activity, and a marked increase in the capacity of the heated solution to precipitate Type XIV antibody (Table IV). On dialyzing the heated solution against repeated changes of distilled water, the dialysate was found to contain 31 mg. of reducing sugar and 4.3 mg. of glucosamine indicating that almost all of the reducing groups and glucosamine liberated by heating had become dialyzable. Part of this material was polysaccharide in nature since, on hydrolysis of the dialysate with 2 N HCl, the reducing sugar increased from 31 to 57 mg. and the glucosamine from 4.3 to 23 mg. Some of the non-glucosamine nitrogen had also become dialyzable since the 23 mg. of total glucosamine in the dialysate accounted for but 1.8 of the 3.2 mg. of nitrogen found. The most striking finding was that the dialysate contained 17 of the 24 mg. of fucose initially present. The concentrated dialysate showed some reactivity with Type XIV antiserum probably due to its content of polymerized material. A small amount of the polysaccharide (1.5 mg.) in the dialysate could be precipitated by alcohol and was shown to precipitate with Type XIV antibody.

Further study of the hog 15 dialysate by paper chromatography after concentration *in vacuo* under CO₂, showed the presence of four components. From the R_F values in both butanol-ethanol-water and collidine-water, fucose and very small amounts of galactose and glucosamine were identified. There was also a component which was essentially immobile (slow) and a component (glycerol from the dialysis bag) which moved more rapidly than the fucose and was seen only when chromatograms were developed with ammoniacal silver (Table V).

Quantitative paper chromatography showed essentially all of the methylpentose to be present in the fucose spot both in the butanol and collidine chromatograms. In butanol this spot contained a small amount of glucosamine which appeared to be in a polymerized form since the amount found was increased on hydrolysis and it is probable that these small quantities of material were included in the fucose spot by uncertainties in cutting the papers.

Both the slow moving and the galactose-glucosamine spots consisted of polysaccharide since marked increases in the glucosamine and reducing sugar occurred on hydrolysis. The non-glucosamine nitrogen (amino acids) also occurred largely in these two fractions. Whether this is bound to the polysaccharides in the dialysate or has been liberated by the heating and merely moves at the same rate as the polysaccharides is uncertain. The unhydrolyzed reducing sugar values for the galactose-glucosamine and the slow spots were 5.7 and

12.4 mg. in butanol and 11.0 and 6.9 in collidine, while the sum of the values for the two spots in each solvent is the same. It is evident therefore, that butanol and collidine effect a different resolution of the polysaccharides present.

The main non-dialyzable fractions from hog 15 (P1, P2) isolated by alcohol precipitation, and showing the enhanced reactivity with the Type XIV antiserum, had essentially similar glucosamine and reducing sugar contents, both hydrolyzed and unhydrolyzed, and an acetyl content and nitrogen similar to that of untreated blood group substances. The fucose content had decreased from 9.2 to 1.5 and 1.7 per cent and the optical rotation had increased from $+10^\circ$ to $+25^\circ$ and $+30^\circ$ for the P2 and P1 fractions respectively.

With the hog 29 substance, the heating was carried out only for 1 hour at pH 1.8 and these milder conditions increased the reducing sugar and glucosamine from 10 to 17 and from 1.8 to 2.4 per cent respectively, corresponding to the liberation of 18.5 mg. of reducing sugar and 1.6 mg. of glucosamine from the 265 mg. of material used. The optical rotation increased from -5° to $+7^\circ$ a change in the same direction and of the same magnitude as that on heating the hog 15 material. Loss of blood group activity and increased reactivity with Type XIV antiserum occurred.

The dialysate contained only 15.3 mg. of reducing sugar and 1.2 mg. of glucosamine, about one-half that found in the case of the hog 15 sample; on hydrolysis these values were increased to 30.6 and 7.4 mg. indicating that some polysaccharide was present. The dialysate contained 17.9 of the 25 mg. of fucose in the starting material.

Results of paper chromatography of the hog 29 dialysate were similar to those with hog 15. Most of the reducing sugar and almost all the methylpentose of the unhydrolyzed concentrated dialysate were found in the fucose spot. The bulk of the polymerized material appeared in the slow component. The galactose-glucosamine component constituted but a minor fraction of the total dialysate and from the R_F value and the finding that the reducing sugar was not increased on hydrolysis, it would appear that it consists chiefly of free galactose. As in the case of the hog 15 dialysate, glycerol from the dialysis sac appeared as a very fast component when chromatograms were developed with ammoniacal silver, but only insignificant quantities of the constituents analyzed for in Table V were present.

The non-dialyzable fractions from the hog 29 substance were similar to those with hog 15 (Table IV), but the yield of non-dialyzable material was much greater due to the milder conditions of hydrolysis and their fucose contents were somewhat higher indicating that only about 60 per cent of the fucose had been split off. The optical rotation had increased from -5° to $+10^\circ$, the latter value being considerably lower than that of the corresponding hog 15 substance. The cross-reactivity of the P1 and P2 substances was much greater than the original material.

Since galactose is the only sugar known to be present in the blood group substances beside glucosamine and fucose, the quantity of galactose liberated can be estimated for the hog 15 substance by subtracting the total glucosamine (23 mg.) plus the fucose calculated as reducing sugar ($17.1 \times 2/3 = 11.4$ mg.) from the total reducing sugar; thus $57 \text{ mg.} - 23 \text{ mg.} - 11.4 \text{ mg.} = 22.6 \text{ mg.}$ of galactose as reducing sugar. Multiplying by $4/3$ the factor for converting the reducing power of galactose to weight gives 30 mg. of galactose. If it is assumed that for each molecule of glucosamine there is one galactose, subtraction ($30 - 23$) shows 7 mg. of galactose over and above that necessary for combination with glucosamine. A similar calculation for hog 29 again using the data in Table V reveals the presence of 14.8 mg. of galactose and 7.4 mg. beyond the amount necessary for combination with glucosamine.

Studies on a hydrolysate of A substance from human saliva W.H.₂ 10 per cent precipitate (10) by paper chromatography in butanol-ethanol-water and in collidine-water served to identify the methylpentose as fucose.

DISCUSSION

The data presented clearly establish (Tables I to III) that blood group A and O substances from hog stomach and the A and B substances from human saliva show an increased capacity to precipitate Type XIV antipneumococcal antibody after heating at 100° at pH 1.5–2.0 although their blood group activity is lost by this treatment. These observations suggest a similarity of structural pattern between these various substances which provides the basis for this behavior; similarities in chemical composition have already been noted (3, 8, 10) and a sample of A substance from human saliva was shown to contain fucose. Study of the chemical changes associated with this mild hydrolysis, in the case of hog 15 (chiefly A substance) and hog 29 (O substance), showed that the predominant change was the liberation of about four-fifths and two-thirds of the fucose respectively in a dialyzable form. In addition, partial depolymerization of the blood group substances occurred as evidenced by the finding of small amounts of free galactose and, in the case of hog 15, of glucosamine as well as considerably larger quantities of polymerized glucosamine-galactose units in the dialysate. In the case of the hog 29 preparation, which had been heated at a somewhat higher pH and for a shorter period than the hog 15 substance, much less partially depolymerized material was found in the dialysate. Appreciable quantities of amino acid nitrogen (non-glucosamine nitrogen) were found in the dialysate and this nitrogen was present in the galactose-glucosamine and slow spots obtained by paper chromatography. Whether this amino acid nitrogen is attached to the polysaccharide in the dialysate has not yet been determined.

The non-dialyzable residues, comprising the bulk of the original blood group substance, showed enhanced reactivity with Type XIV antiserum and were

devoid of blood group activity. They had essentially the same analytical composition as the original blood group substance except for the marked reduction in methylpentose content and the increase in optical rotation (Table IV). This is not surprising, however, since it is doubtful whether the elimination of the fucose in these two instances, would significantly affect the glucosamine, reducing sugar, nitrogen, or acetyl values.

The correlation between the immunochemical and the chemical data establishes a portion of the structure of the blood group substances. The Type XIV specific carbohydrate (SXIV) and the hog blood group A and O substances have been shown to consist of *N*-acetylglucosamine and galactose residues. By virtue of the cross-reaction of the blood group substances with Type XIV antiserum, it would seem reasonable that both the SXIV and the blood group substances possess in the basic chain a series of *N*-acetylglucosamine-galactose residues; the sequence in the various substances not necessarily being identical, but giving rise to patterns with certain similarities of structure at various points on the chain, much as in the case of the structures proposed for SIII and SVIII to explain their cross-reactivity with their heterologous antipneumococcal horse sera (20, 21). The blood group substances, unlike SXIV, contain fucose residues (8, 9, 15) and by methylation studies, Bray, Henry, and Stacey (9) have shown these to be present as end groups. If these fucose end groups were attached to the main *N*-acetylglucosamine-galactose chain at various points, they would project outward and might very well prevent the cross-reacting groupings from combining with antibody. Removal of these fucose residues might either provide additional cross-reacting sites on the main chain or might make possible a closer approach of the antibody molecule to the reactive groupings on the main chain and thereby increase the extent of the cross-reaction as has been observed. The fact that the fucose may be easily removed without effecting appreciable hydrolysis of the main galactose-glucosamine chain is further evidence that the fucose does, indeed, occur as end groups and is not an integral part of the main polysaccharide chain. Further examination of the data reveals that, for both the hog 29 and hog 15 substances, hydrolysis resulted in the splitting off of almost identical amounts of fucose, of galactose beyond that necessary for combination with glucosamine, and of amino acid nitrogen. Data of this nature indicate that the mild conditions used in these experiments produce an orderly cleavage of blood group A and O substances. Fucose and galactose are split off in a ratio of roughly 3:1, and a definite proportion of the non-glucosamine nitrogen (amino acids) is liberated suggesting that these substances form an easily hydrolyzed unit or units attached to the main polysaccharide chain. It further indicates that there are two types of non-glucosamine nitrogen (amino acids). The labile amino acids may possibly be responsible for conferring blood group activity on the galactose-glucosamine polysaccharide, since as will be demonstrated in the following paper, the fucose

(or at least some of it) is not necessary for blood group activity. Further studies will be needed to clarify these relationships.

The structural relations thus far proposed are in agreement with the data. However, they do not provide any explanation of the previous report (1) that various samples of blood group A and O substances from individual hog stomachs show different capacities to react with Type XIV antiserum. This will be treated in the accompanying paper (22) in which it is demonstrated that the hog blood group substances vary in their fucose content and that the capacity of the substance to cross-react with Type XIV antiserum is inversely related to its fucose content, providing strong additional evidence for the structural relations proposed.

SUMMARY

1. The effect of heating at varying pH on the cross-reactivity of hog blood group A and O substances with Type XIV antibody has been investigated. The hydrolysis of blood group A, B, and O substances from hog and human sources at pH 1.5-1.8 resulted in destruction of blood group activity and a marked increase in cross-reactivity with Type XIV antipneumococcus horse serum.

2. Analysis revealed the liberation of reducing sugar, most of which was dialyzable. It was further shown that the major part of the reducing sugar was fucose with small amounts of free and polymerized galactose and glucosamine. Non-glucosamine nitrogen, probably amino acids, also was found in the dialysate.

3. Paper chromatographic separation of the concentrated dialysate confirmed the presence of fucose and showed that the majority of the galactose and glucosamine occurred in polymeric form.

4. Quantitative analysis of the substances isolated from the paper showed good recovery of the various components, indicating that practically all of the material in the dialysate could be accounted for.

5. The methylpentose of human blood group A substance was identified as fucose.

6. The structure of the blood group substances is interpreted in terms of these results.

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IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

VIII. THE METHYLPENTOSE CONTENTS OF HOG AND HUMAN BLOOD GROUP A AND O SUBSTANCES AND THEIR RELATIONSHIP TO CROSS-REACTIVITY WITH TYPE XIV ANTIPNEUMOCOCCUS HORSE SERUM*

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The presence of *l*-fucose in purified blood group A and O substances from hog gastric mucosa has been established in three laboratories (1-3). In the preceding paper (4) it was shown that heating at pH 1.5-1.8 liberated most of the fucose from the polysaccharide. Since the heating at pH 1.5-1.8 also increased the ability of the blood group substances to cross-react with Type XIV antipneumococcus serum, it was suggested that this increased cross-reactivity resulted from the removal of fucose residues projecting as end groups from the main polysaccharide chain. It had previously been demonstrated (5) that preparations of hog blood group A substances from individual hog stomach linings varied in their capacity to precipitate Type XIV antibody although they were identical in blood group A activity and it was considered of interest to investigate the fucose content of these substances to determine whether the fucose was responsible for this variation. For this purpose the newly developed specific color reaction for methylpentoses (6) was employed and the methylpentose contents of the various hog and human blood group substances determined. These values are assumed to represent fucose which is the only methylpentose known to be present in the blood group substances (1-4). Considerable variation in the methylpentose contents of the individual samples of blood group substances was found. Among the hog blood group A and O substances the methylpentose content varied inversely with the degree of cross-reactivity with Type XIV antipneumococcal serum.

EXPERIMENTAL

The individual preparations of hog and human blood group substances are identical with those described in the previous studies (2, 7, 8).

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Methylpentose was determined (6) by slowly adding to 1 ml. of an ice cold solution of the blood group substance containing between 3 and 10 $\mu\text{g.}$ of methylpentose, 4.5 ml. of sulfuric acid solution (6 parts of acid to 1 part of water by volume). The addition of the acid was made slowly and with constant shaking in an ice bath to prevent a rise in temperature. The tubes were transferred to a water bath at room temperature for a few minutes, then to a vigorously boiling water bath, which should not cease boiling when the tubes are inserted. After being heated for exactly 3 minutes, the tubes were placed in a water bath at room temperature. 0.1 ml. of a 3 per cent solution of cysteine hydrochloride (prepared weekly and stored under refrigeration) was added and the tubes were thoroughly mixed. After 2 hours the absorption was measured in a Beckman spectrophotometer at 3960 and 4300A, the difference being directly proportional to the methylpentose content of the solutions (6).

TABLE I
Fucose Content of Hog Blood Group A and O Substances

Blood group A substance	Fucose	Cross- reactivity*	Blood group O substance	Fucose	Cross- reactivity*
	<i>per cent</i>	$\mu\text{g. N}$		<i>per cent</i>	$\mu\text{g. N}$
Hog 3	6.8, 6.6	72	Hog 6	6.5	65
5	8.2, 8.3		13	6.8	64
8	7.3	72	19	11.5	23
10	8.9	19	25	11.2, 14.2	23
14	7.7, 7.8		27	7.3, 7.4, 7.8	80
15	9.9, 9.2	35	29	9.5, 8.8, 8.9, 8.3, 8.1	63
16	9.6	39	32	5.7	118

* $\mu\text{g.}$ of N precipitated by 500 $\mu\text{g.}$ of blood group substance from 0.5 ml. of Type XIV antipneumococcus horse serum H635 (*cf.* 5).

All analyses for fucose carried out on samples of 50 or 100 $\mu\text{g.}$ blood group substance.

RESULTS

That wide differences exist in the fucose content of the individual blood group A and O preparations may be seen from Tables I and II. The hog substances vary from 6 to about 13 per cent and the human preparations exhibit a somewhat similar variation; *i.e.*, 9 to 16 per cent. The average methylpentose content of the human blood group substances (approximately 13 per cent) is higher than that of the hog substances (approximately 9 per cent). Table I also gives the amount of total nitrogen precipitated from 0.5 ml. of Type XIV antipneumococcal serum H635 by 500 $\mu\text{g.}$ of the various hog blood group A and O substances; with few exceptions data were previously reported (5). Fig. 1 shows a plot of the amount of nitrogen precipitated by 500 $\mu\text{g.}$ of hog substance from 0.5 ml. of Type XIV serum against the percentage of methylpentose. The numbers represent the number of the hog blood group preparation. It is evident that there is an inverse relationship between the methylpentose content of the various hog A and O substances and the cross-reactivity. A similar type of curve was obtained when the amount of nitrogen precipitated by 250 $\mu\text{g.}$ of blood group substance was plotted against the fucose content of the hog A and O substances.

No such relationship was evident when the data (5) on the cross-reactivity of the human blood group substances were compared with their methylpentose contents (Table II).

TABLE II
Fucose Content of Human Blood Group A, B, and O Substances

Blood group A substance	Fucose		Blood group B substance	Fucose		Blood group O substance	Fucose	
	per cent	Cross- reactivity* μg. N		per cent	Cross- reactivity* μg. N		per cent	Cross- reactivity* μg. N
<i>Saliva</i>			<i>Saliva</i>			<i>Saliva</i>		
W.H. ₁ 10 per cent ppt.	14.0	55	S.E. 10 per cent ppt.	11.6	65	Bd. phenol-insol.	8.8	
W.H. ₁ phenol-insol.	13.9	81	S.E. phenol-insol.	13.0	56	Bd. digest of water-insol., phenol-insol.	12.4	
W.H. ₂ 10 per cent ppt.	15.4	102				Bd. 10 per cent ppt.	8.4	42
W.H. ₂ digest of water-insol. 10 per cent ppt.	12.9					Bd. not digested phenol-insol.	11.2	
W.H. ₂ undigested phenol-insol.	12.3	58				Bd. digest of water-insol. 10 per cent ppt.	9.2	
G.C. 10 per cent ppt.	16.0					F.P. 10 per cent ppt.	14.6	105
G.C. phenol-insol.	13.0	60				F.P. digest of water-insol. phenol-insol.	18.4	
B.K. phenol-insol.	12.4	86				F.P. digest of water-insol., 10 per cent ppt.	13.8	
B.K. 10 per cent ppt.	11.0	81				F.P. not digested phenol-insol.	11.2	
A.K. 10 per cent ppt.	16.4					Human stomach 5	12.6	72
M.S. 10 per cent ppt.	11.4							
Amniotic fluid I phenol-insol.	9.6	125						
Amniotic fluid I 10 per cent ppt.	13.3							
Human stomach 1	9.0	65						
Human stomach 2	11.0	62						
Human stomach 3	16.0	29						
Human stomach 4	11.5	50						

* μg. N precipitated by 500 μg. of blood group substance from 0.5 ml. of Type XIV anti-pneumococcal horse serum H635.

DISCUSSION

The inverse relationship between cross-reactivity with Type XIV antiserum and fucose content provides further information about the structure of hog

blood group A and O substances. It is in accord with the concept advanced in the preceding paper that fucose residues of the blood group substances project as end groups, from a basic chain of *N*-acetylglucosamine-galactose units and that these fucose residues reduce the cross-reactivity of the substances either by covering reactive groups on the main chain or by preventing antibody molecules from approaching sufficiently close to the main chain to react with free cross-reactive sites. The present finding that the individual hog A and O substances may have different fucose contents (Table I) and that their capacity to cross-

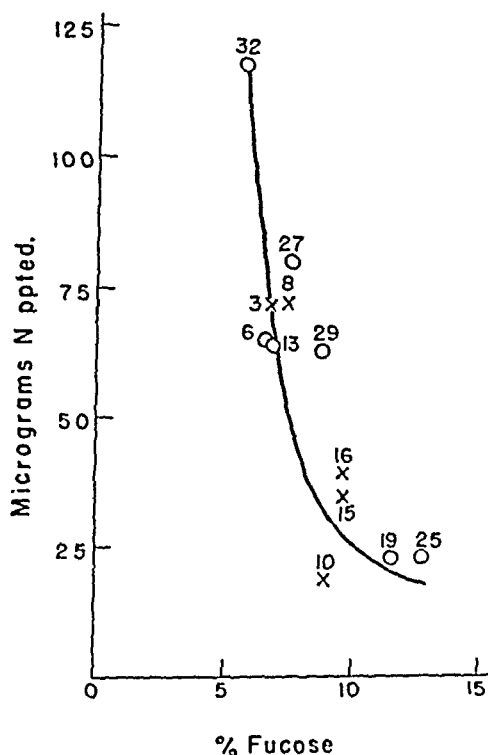


FIG. 1. Relation between fucose content and cross-reactivity of hog A and O substances with Type XIV antipneumococcal horse serum H635. X = hog A substances; O hog O substances. Ordinate = micrograms N precipitated by 500 μ g. blood group substance from 0.5 ml. antiserum.

react with Type XIV antibody varies inversely with their fucose content (Fig. 1) provides strong support for this concept and in addition accounts completely for the variation in cross-reactivity of hog blood group A preparations of identical blood group A activity (5). It would appear from the curve in Fig. 1 that the ratio of fucose residues to the total number of residues in the main chain reaches a limit, which, from the slope of the curve, is probably not much greater than about 15 per cent, and corresponds to about one fucose side chain to about every three or four hexose residues in the main chain.

Since the blood group A substances, which have been shown to be identical in blood group A activity, may vary widely in their fucose contents, it is evident

that a considerable proportion of the fucose is not essential for blood group activity. For instance hog 16 with 9.6 per cent of fucose showed the same capacity to precipitate anti-A and the same purity based on glucosamine analyses of specific precipitates as did hog 3 with but 6.6 per cent fucose, hog 8 with 7.3 per cent, hog 5 with 8.3 per cent fucose, or hog 10 with 8.9 per cent fucose (7, 2). Furthermore, the relation between cross-reactivity and fucose content does not depend upon whether the individual blood group substance exhibits A activity or O activity or both, since all of the hog substances lie on the same curve (Fig. 1) indicating that blood group specificity is not determined by this variation in fucose content. Analyses of specific precipitates of A substance and anti-A (7) for their fucose content should provide further information on the relationship of the fucose to the blood group substances.

No correlation between cross-reactivity and fucose content was evident for the human blood group substances. This is not surprising since the substances appear to show a greater complexity than do the hog substances. The individual human blood group A substances showed greater variability than did the hog substances in their analytical properties and in their blood group activity. Furthermore, the substances from human saliva after peptic digestion, unlike the hog substances, occurred in two fractions, a phenol-insoluble and a phenol-soluble fraction from which the blood group substance could be precipitated by 10 per cent alcohol.

SUMMARY

It has been possible, by employing a new color reaction for methylpentoses, to determine the fucose content of individual hog and human blood group A, B, and O substances. The data indicate an inverse correlation between fucose content of the hog A and O substances and ability to cross-react with Type XIV antipneumococcus serum. The human A, B, and O substances display no correlation between their fucose content and ability to cross-react with Type XIV antipneumococcus serum.

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POLIOMYELITIS IN THE CYNOMOLGUS MONKEY

IV. FURTHER OBSERVATIONS ON EXPOSURES CONFINED TO THE STOMACH AND INTESTINES, WITH NOTES ON THE FECAL EXCRETION OF VIRUS*

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In an earlier publication (1) we showed that non-traumatic exposure of the stomach and intestines to poliomyelitis virus (*Per* strain) without simultaneous exposure of the oropharynx and upper esophagus failed to induce infection in a series of 26 *cynomolgus* monkeys; while the same strain administered by simple feeding, which involved simultaneous exposure of both the pharyngeal and gastrointestinal levels, had been shown by Sabin and Ward (2) to induce infection in 40 per cent of 15 tests. These observations strongly suggested that the gastrointestinal mucosa, as compared with the oropharyngeal, was relatively impervious to poliomyelitis virus, casting doubt on the validity of the concept that the gastrointestinal tract is the usual portal of entry in this disease.

This earlier study was an exploration which we desired not only to repeat with another strain of virus, preferably recently isolated, but also to amplify and expand. In particular we were anxious to observe the appearance and persistence of virus in the stools which is one of the conspicuous features of human poliomyelitis and which has not as yet been adequately explained. In the first study, probably because of the inadequacy of the technique then available for virus recovery from stools, we failed to detect virus in the stools of any of the treated animals tested. We also desired to discover whether intestinal exposure alone, without infection, would set up a state of resistance to later intracerebral inoculation. It will be recalled that in the previous study (3), during which a series of other exposures to virus followed the gastrointestinal, 4 of 5 of the surviving animals displayed a limited resistance to later intracerebral inoculation.

The present experiments concern 18 *cynomolgus* monkeys fed by capsule by the same method as was used in the earlier study, and the results will be related to those of simple feeding of the same strain of virus, which we are reporting elsewhere (4).

EXPERIMENTAL METHODS

Arrangement of Animals.—All monkeys used in this experiment were *M. irus* (*cynomolgus*) from the Philippine Islands, received on Oct. 7, 1946. On Oct. 23, 22 normal, healthy monkeys

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were separated into 3 groups of 6 each for the experiment, and one group of 4 as controls. The animals of each group were in adjacent cages, containing 2 each, but the groups were separated by a distance of at least 3 feet.

Preparation of Virus.—The *Cam* strain, isolated in this laboratory in July, 1945, from stools of a mild paralytic human case of poliomyelitis, was used throughout, in a mixture of the first five passages. This strain has produced infection, frequently in mild form, in 87 per cent of *rhesus* and 94 per cent of *cynomolgus* monkeys inoculated intracerebrally. Of 6 monkeys fed this strain mixed with the regular diet, 3, or 50 per cent, succumbed to the disease (4). The variability of clinical manifestations has been reminiscent of that seen in outbreaks of the human disease, some of the infections being non-paralytic, some monoplegic, a few bulbar, and only rare ones, fulminant.

A mixture of histopathologically proved, severely involved segments of spinal cord from 10 paralyzed *rhesus* monkeys sacrificed 5 to 15 months previously was used as virus. 45 gm. of tissue, which had been stored on dry ice, was emulsified in a Waring blender with 70 cc. of cottonseed oil. The same suspension was used throughout, being stored on dry ice between feeding periods. Enough suspension for a single feeding period was thawed when needed, and kept in the refrigerator until used.

Preparation and Administration of Capsules Containing Virus.—The required number of number 4 gelatin capsules for one feeding only were filled with well mixed suspension and immediately coated with a heavy layer of crisco.¹ There was no visible contamination of the outside of the capsules while filling or coating. Three capsules were placed in a specially constructed curved metal cannula, which was then placed in the esophagus of an unanesthetized monkey. The capsules were forced out by means of a closely fitting metal obturator. In a few instances, unopened capsules returned to the mouth as a result of gagging, but were immediately reintroduced into the esophagus. No subsequent regurgitation was observed. Monkeys were given capsules before the morning meal on 3 successive days and received approximately 1.2 gm. of cord (wet weight) in this period. The dates of feeding and the interval between feedings in those animals exposed more than once are stated below.

Intracerebral Challenge.—The animals of each group were tested for possible refractoriness by intracerebral (intrathalamic) inoculation approximately 5 weeks after the last feeding for the particular group. Fourth passage *Cam* virus was used for the purpose, in 20 per cent concentration for group I, and 10 per cent concentration for groups II and III. The 4 control animals were inoculated intracerebrally at the same time and with the same amounts as group III, which also served as a test of the activity of the virus.

RESULTS

Group I. One Series of Feedings.—Oct. 30, 31, Nov. 1, 1946: 6 monkeys (C1-10, C1-13, C1-14, C1-15, C1-16, C1-27) were given 3 capsules each day. Nov. 11, 1946: C1-14 became paralyzed and was sacrificed. Dec. 2, 1946: the remaining 5 animals were challenged by intrathalamic inoculation of 0.8 cc. of 20 per cent *Cam* strain (4th passage). Dec. 8-12, 1946: all 5 became paralyzed and were sacrificed.

Protocol, C1-14.—Oct. 30, 31, Nov. 1, 1946: 3 capsules daily were administered, as above noted. Nov. 4-9, 1946: no symptoms were noted. Virus was recovered from pooled stools of the entire group up to Nov. 3, 2 days after feeding, and from stools of C1-14 pooled with C1-15, collected during the period Nov. 4-9. Stools from all other animals were negative during the latter period. Nov. 10, 1946: the temperature was 103.0°, about a degree above normal for the animal. No clinical symptoms were observed. Nov. 11, 1946: the tempera-

¹ Crisco is stated by the manufacturer, Proctor and Gamble, Cincinnati, to be "a pure vegetable shortening."

ture was 103.3°. There were slight tremor, slight overbreathing, easy fatigability, and paralysis of the left arm. No bulbar symptoms were noted. The animal was sacrificed immediately for histological study. Colon contents were removed and found to contain virus. It thus seems fairly certain that virus recovered from the stool pool from C1-14 and C1-15 came from C1-14.

Microscopic Examination.—The olfactory bulbs and tertiary olfactory centers were normal throughout. Minimal PvI and PrI² were found in the hypothalamus, but only PvI in the thalamus. Lesions in the midbrain were confined to the reticular formation and the right mesencephalic nucleus of the fifth cranial nerve. Comparable lesions were found in the locus coeruleus on the same side. In the pons, involvement of the reticular formation increased in intensity from minimal rostrally to moderate caudally. The main sensory nucleus of the fifth nerve showed minimal lesions on the left side only, while the nucleus of the spinal tract of the fifth nerve was questionably involved on the left and moderately so on the right. Lesions in the right spinal V nucleus were more marked than elsewhere in the pons, with the exception of the reticular formation. Minimal lesions were found in both vestibular and both motor VII nuclei. In the medulla, moderate involvement was found in the reticular formation and the left hypoglossal nucleus, minimal in the olive, and none elsewhere.

Scattered lesions of varying severity were found throughout the spinal cord, more marked in the cervical regions. Several levels were free of lesions excepting an occasional small PvI. The sympathetic columns showed no involvement of nerve cells at any level; at T₁ there were a few scattered infiltrating microglial cells, and at T₁₁ there was a small PvI with PrI medial to the column with a few infiltrating cells in the column itself apparently extending from the adjacent lesion.

Of the peripheral ganglia of the cranial nerves, only the Gasserians were significantly involved. Moderate to marked PrI with marked neuronophagia was found on both sides. It should be noted in this connection that the central sensory connections of the V nerve were only minimally involved on the left side and moderately so on the right where a large well isolated focus was found in the spinal V nucleus in the pons. The greater severity of lesions in the peripheral component of the fifth cranial nerve system suggests to us that lesions in the Gasserian ganglia were of centripetal origin. Other cranial nerve ganglia contained only minimal infiltrative foci of questionable significance, or none whatsoever.

The celiac ganglion showed a few infiltrative lesions but no neuronophagia. One superior cervical sympathetic ganglion showed marked lesions with probable neuronophagia; the other, only minor lesions. The stellate, thoracic sympathetic, lumbar sympathetic, and spinal ganglia were not studied.

The distribution of lesions in this animal was compatible with and rather suggestive of entry of infection through the nasopharynx by way of the fifth cranial nerve; for entry *via* the sympathetics of the head and intestinal areas the evidence is much less suggestive but not wholly incompatible since lesions were observed in their peripheral components (ganglia) and some minor ones were found at least adjacent to their central stations (sympathetic columns of the cord) at two of the corresponding levels. While it is true that contamination of the oropharynx was carefully avoided in the original administration of virus, secondary contamination from infected stools could not be avoided, and may explain the apparent oropharyngeal portal of entry. The possibility of unobserved regurgitation of fed virus cannot be entirely excluded.

² PrI, parenchymal infiltration. PvI, perivascular infiltration.

Group II. Two Series of Feedings.—Oct. 30, 31, Nov. 1, 1946: 6 animals (C1-17, C1-23, C1-28, C1-29, C1-30, and C1-33) were given 3 capsules each day. Dec. 2, 3, 4, 1946: all animals having remained well, they were again given 3 capsules each day. Jan. 6, 1947: all 6 having remained well, their susceptibility was challenged by intrathalamic inoculation of 0.8 cc. of 10 per cent *Cam* virus (4th passage). Jan. 12-15, 1947: all 6 became paralyzed and were sacrificed.

Group III. Three Series of Feedings.—Oct. 30, 31, Nov. 1, 1946: 6 animals (C1-18, C1-19, C1-20, C1-21, C1-22, and C1-24) were given 3 capsules each day. Dec. 2, 3, 4, 1946: all having remained well, they were again given 3 capsules each day. Jan. 6, 7, 8, 1947: all having remained well, they were again given 3 capsules each day. Feb. 14, 1947: all having remained well, their susceptibility was challenged by intrathalamic inoculation of 0.8 cc. of 10 per cent *Cam* virus (4th passage). Feb. 20, 1947: C1-20 died of intercurrent causes, without observed symptoms of poliomyelitis, but showed typical lesions in the spinal cord. Feb. 21-25, 1947: the remaining 5 animals became paralyzed and were sacrificed.

Controls.—Feb. 14, 1947: 4 animals (C1-11, C1-25, C1-26, and C1-34) were housed throughout the experiment in the same room as those exposed to virus. They were inoculated intrathalamically on this date with 0.8 cc. of 10 per cent *Cam* virus, (4th passage). Feb. 21-25, 1947: all 4 became paralyzed and were sacrificed.

Stool Collection and Preparation.—Stools were collected daily, kept separate with regard to cage (2 monkeys per cage), but pooled by time interval, and stored on dry ice until prepared for inoculation.

All stools passed during the 48 hours before the first administration of virus were saved as normal controls. Feeding period stools were collected from the afternoon of the day of the first capsule feeding until 48 hours after the last; *i.e.*, until all ingested virus could reasonably be assumed to have left the alimentary tract. Thereafter, stools were collected in weekly pools from 9 a.m. Monday morning until 12 noon Saturday. An interval of from 1 to 3 days elapsed between the end of the "feeding period" collection and the beginning of the next collection.

Heavy suspensions, in distilled water, of the stools from each cage were prepared in a Waring blender. After preliminary centrifugation for about $\frac{1}{2}$ hour at 1600 R.P.M., the turbid supernate was clarified by centrifugation at 18,000 R.P.M. for 20 minutes. The clear supernates were then pooled by groups and centrifuged for 4 hours at 18,000 R.P.M. in the presence of 10 per cent normal monkey serum by the method developed in this laboratory (5). The pellets were resuspended in saline and stored on dry ice. While toxic reactions to intracerebral inoculation of material prepared by this technique are very few, they have occasionally occurred. Further high speed centrifugation at 18,000 R.P.M. for about 20 minutes after thawing and just before inoculation results in a water-clear, colorless suspension, completely devoid of toxic effects.

One monkey each was inoculated intracerebrally with stool concentrate from each group of experimental animals. When the result was equivocal, a second animal was inoculated with the same material. Supernate was saved from the original suspensions prepared, so that pools could be broken down to individual cages if necessary. All animals remaining free of symptoms for 4 weeks were sacrificed for microscopic examination of the brainstem for evidence of inapparent infection.

Results of Tests for Virus in the Stools

These are summarized in Table I. Virus was recovered from all groups during the feeding periods with the exception of group III at the time of the second feeding. Only one interim pool was positive, that of group I, collected

6 to 11 days following the first feeding. This group contained C1-14, the animal which developed fever the 1st day and paralysis the 2nd day after the pool was closed. Stools from each of the 3 cages housing the 6 monkeys of this group were then tested separately; the only positive result was from the cage of C1-14 and C1-15. C1-14 was sacrificed and virus recovered from its intestinal contents. C1-15 remained in the group from which stool was collected beginning the day on which C1-14 succumbed (C1-14 having been removed and the cage cleaned just before the collection began), and the pools

TABLE I
Virus in Stools after Capsule Feeding

Days of experiment when stools were collected	Group I	Group II	Group III	Controls
Control (before feeding)	0	—	—	—
1-5 (1st feeding period, 1-3)	+	+	+	—
6-11	+	0	0	0
13-16	0	0	0	0
20-25	0	—	—	—
27-32	—	—	—	—
34-38 (2nd feeding period, 34-36)		+	0	0
41-46		0	0	0
48-53		0	?	—
55-61		—	—	—
62-68			—	—
69-73 (3rd feeding period, 69-71)			+	0
76-81			0	0
83-88			0	—

+, test positive. ?, questionable inapparent infection in animal inoculated with stool.
0, test negative. —, stool not tested.

* Virus in this pool was traced to C1-14, the animal which developed poliomyelitis (see protocol).

were negative during this and the following week. There can therefore be little doubt that the virus detected in the 6 to 11 day pool came from C1-14.

A very questionably positive test for virus was found in the animal subinoculated with stool from group III during the 2nd week after the second feeding. No clinical signs of infection were noted. Scattered PvI and rare PrI, but no neuronophagia, were found in the brainstem from the thalamus down to the upper medulla. A *cynomolgus* monkey later inoculated with the same material remained well.

DISCUSSION

Our present experiments, as well as those previously reported, show that when virus is administered in such a way as to expose only the lower alimentary

tract, avoiding contamination of the oropharynx as far as possible, poliomyelitic infection rarely supervenes. In the present series of 18 *cynomolgus* monkeys fed a total of 36 times by capsule inserted into the esophagus, only one case of poliomyelitis occurred; and in the previous series of 26 animals, each fed once by the same method, no cases occurred. Thus, in 44 animals with a total of 62 capsule-feedings, poliomyelitis resulted only once. In sharp contrast with this, is the high proportion of takes after simple oral feeding with oropharyngeal as well as gastrointestinal exposure (2, 4).

Granting that in the single case of poliomyelitis after capsule feeding and in one of the cases of simple feeding (4) entry may have occurred through the intestine (proof of which is not conclusive), the evidence substantiates our earlier conclusions that the oropharynx is a more favorable site for entry of

TABLE II
Results of Simple Feeding as Compared with Capsule Feeding

	Simple feeding			Capsule feeding		
	<i>Per virus</i>	<i>Cam virus</i>	Both	<i>Per virus</i>	<i>Cam virus</i>	Both
Monkeys tested.....	15(2)	6	21	26	18	44
Positive.....	6	3	9	0	1*	1
Positive, <i>per cent.</i>	40	50	43	0	5.6	2.3

* Distribution of lesions in this animal suggests oropharyngeal entry rather than intestinal.

poliomyelitis than is the intestine, and that the latter is relatively impervious to the virus.

This conclusion has, we believe, an anatomical basis which would apply to man as well as to the lower primates. If, as we have elsewhere suggested (6), poliomyelitic infection enters the body through the superficial fibers (telodendria) of the regional nerve supply where virus is deposited, the reason for the failure of virus in the intestinal canal to cause infection may be looked for in the anatomical arrangements of its terminal nerve fibers. The absence of sensation for touch and pain excepting in the mesentery (7) (Vater-Pacinian receptors) indicates that there are no exteroceptive endings in the epithelium; and the histological studies of Cajal (8) and of Oshima (9) show clearly that such nerve fibers of any kind as approach the epithelial surface penetrate no farther than to the subepithelial level; they would, therefore, appear to be protected from direct contact with intestinal contents and so with virus in the contents, unless the integrity of the epithelial layer were broken. Although further study of the point is desirable it appears that in the pharynx and mouth the telodendria of regional nerves, at least of the trigeminal (10), actually do penetrate between the epithelial cells to the surface and thus are in a position to afford direct contact with virus lodged on the surface. If this is correct,

the relative ease with which infection can enter through the oropharyngeal surfaces obtains an anatomical explanation. The normal oropharynx would, so far as its superficial nerves are concerned, present an open door to poliomyelitis virus but the same would not be true of the lower alimentary tract, where trauma or ulceration of the surface would presumably be required for entry.

Another mode of invasion through the surface, by lymphatic absorption, deserves consideration. Since Flexner and Clark's (11) original observations it has been known that in poliomyelitis, virus is not uncommonly present in the tonsils and sometimes in other regional lymph nodes, including the cervical and mesenteric (12). The possibility therefore exists that virus might make its primary entry by the lymphatics and after deposit in lymph nodes make contact with nerve elements to produce the initial infection. The present experiments argue against such a theory, since lymphatic absorption is probably much greater in the intestine than elsewhere in the body, whereas infection *via* the intestine occurred no more than twice despite heavy exposures.

The conditions under which virus appeared in the stools, and then disappeared or persisted deserve attention. It appeared quite regularly during the immediate periods after feeding, and, with a single exception, disappeared thereafter. Obviously, the intestinal mucosa was in all instances thoroughly exposed and the virus had an excellent opportunity to implant itself upon and to multiply in the intestinal epithelial cells if they are in fact potential hosts to the virus, as is suggested by the comments of Evans and Green (13). Nevertheless, the only instance in which virus was recovered from the stools after the immediate feeding period occurred in the animal that developed manifest poliomyelitis. The inference is therefore at least tentatively permissible that intestinal epithelium is not a suitable host for this particular virus, and that it appears in the stools of subjects of the disease by a process of excretion from infected nerve tissue. Preliminary observations on the mechanism of excretion of poliomyelitis virus, pointing in that direction have already been published by us (14), and others will, it is expected, appear later on.

Tests for resistance to poliomyelitis virus following gastrointestinal exposure without infection were uniformly negative. The method of intracerebral inoculation with large doses of virus is obviously the severest possible test, and would fail to reveal such lesser degrees of immunity as might conceivably have occurred.

SUMMARY

36 exposures of the stomach and intestines of 18 *cynomolgus* monkeys to large doses of poliomyelitis virus by a method designed to avoid simultaneous exposure of the oropharynx and upper esophagus induced poliomyelitis in only one instance.

These observations are to be compared with a previous study in which ex-

posures of the entire alimentary tract including oropharynx and upper esophagus by simple feeding of comparable amounts of the same strain resulted in poliomyelitis in half of the test animals.

In the capsule-fed animals virus regularly appeared in the stools during and immediately after the feeding periods but disappeared thereafter excepting in the single case of poliomyelitis, in which it persisted.

No evidence of resistance to subsequent intracerebral inoculation was observed in the uninfected capsule-fed animals.

CONCLUSIONS

1. The gastrointestinal mucosa appears to be relatively impermeable to poliomyelitis virus. On the experimental evidence as well as on anatomical grounds we regard the gastrointestinal mucosa as an infrequent and exceptional portal of entry.

2. The experiments suggest that persistence of virus in the stools in poliomyelitis is due to excretion, presumably originating in infected nervous tissue, rather than to multiplication of the agent on intestinal epithelium.

3. Exposure of the gastrointestinal epithelium without resulting infection failed to produce immunity to intracerebral inoculation.

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THE EFFECT OF SPHINGOMYELIN ON THE GROWTH OF TUBERCLE BACILLI

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The enhancing effect of the synthetic wetting agents Tween 40, Tween 60, and Tween 80 on the growth of tubercle bacilli is due in part to the fact that these water-dispersible esters constitute for the bacteria a non-toxic source of palmitic, stearic, or oleic acid. Indeed, enhancement of growth can also be observed with the sodium soaps of these same fatty acids, as well as of linoleic, linolenic, and arachidonic acids, provided that the bacilli are protected against the toxic action of these soaps, for example by the addition of serum albumin to the medium (6).

In addition to long chain fatty acids, there exist in tissues other lipids which enhance the growth of tubercle bacilli (1, 2, 5, 10). Further study of this problem has led to the recognition that, in this respect, sphingomyelin is more active than any of the other naturally occurring phospholipids and cerebrosides tested in our laboratory. It will be shown in the present paper that the addition to simple culture media of sphingomyelin prepared from various tissues markedly increases the density of growth within a short period of incubation and also facilitates initiation of growth from small inocula, even in the absence of serum albumin or other protein.

EXPERIMENTAL

All experiments described in the present report were carried out with a virulent culture of the strain of human tubercle bacillus H37Rv recently recovered from infected mouse lung and maintained in the Tween-albumin medium described elsewhere (8). The 10 day old culture used for inoculation consisted of finely dispersed bacilli and contained approximately 0.35 mg. bacillary cells (dry weight) per cc. of medium.

The basal medium used in the experiments had the following composition:

KH_2PO_4	1.0	gm.	} heat in 100 cc. distilled water to dissolve
$\text{Na}_2\text{HPO}_4 \cdot 12 \text{ H}_2\text{O}$	6.3	"	
Asparagine	1.0	"	
Add:			
Distilled water	850 cc.		
Enzymatic digest of casein ..	0.5	gm.	(10 cc. of a 5 per cent solution in distilled water)
ZnSO_4	0.001	"	(1 cc. of a 0.01 per cent solution in distilled water)
Ferric ammonium citrate	0.005	"	
$\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$	0.01	"	(1 cc. of a 1 per cent solution in distilled water)

CaCl ₂	0.0005 gm.	(1 cc. of a 0.05 per cent solution in distilled water)
CuSO ₄	0.0001 "	1 cc. of a 0.01 per cent solution in distilled water)
Adjust pH to 6.5		

It was distributed in 3 cc. amounts in pyrex glass tubes 25 mm. in diameter. For reasons given in an earlier publication, aluminum caps were used instead of cotton plugs (7). The other ingredients were added, as indicated in the text, either before autoclaving or aseptically as sterile solutions.

Serum albumin was the fraction V of bovine plasma. It was dissolved in a concentration of 5 per cent distilled water. The solution was sterilized by filtration through a porcelain candle.¹

The non-ionic wetting agent Triton A20² was added to the medium in a number of tests. This wetting agent is a heat-stable arylalkyl polyether of phenol which disperses the cultures of tubercle bacilli without increasing the yield of growth. Its properties will be further discussed in a subsequent publication.

Most of the preparations of sphingomyelin, cerebroside, lignoceric amide, lignoceric acid, and sphingosine used in the present study were obtained from the chemical collection of the late Dr. P. A. Levene maintained at the Rockefeller Institute. The chemical structure and properties of these substances as well as the methods involved in their preparation have been described in references 9 and 11. A sample of pure lung sphingomyelin was generously supplied by Dr. S. J. Thannhauser of Joseph H. Pratt Diagnostic Hospital, Boston; this material had been freed of contaminating dipalmityl glyceride by the technique described in reference 12.

All preparations of lipids were dispersed in distilled water starting from chloroform solutions whenever necessary. Sphingomyelins and cerebroside were sterilized by three consecutive heatings at 80°C., although more recent tests indicate that their effect on the growth of tubercle bacilli is not appreciably modified by autoclaving in the basal medium.

All lipids imparted a certain degree of opalescence to the medium. It was observed that complete dispersion of sphingomyelin, resulting in a clear medium, could be obtained by adding to the medium the wetting agent Triton A20 (see above) in a final concentration of 0.01 to 0.02 per cent.

Effect of Sphingomyelin and Cerebroside Preparations on the Growth of Tubercle Bacilli.—The yield of bacilli within a given period of incubation and the ability of the medium to allow proliferation of very small inocula have been used as criteria in the following experiment to compare the growth-promoting properties of sphingomyelins and cerebroside and of serum albumin.

The preparations of sphingomyelin and cerebroside to be tested were added in 0.3 cc. amounts of emulsions of various concentrations to 3 cc. of the basal medium. Albumin (0.3 cc. of 5 per cent solution), or water, was used for the control media. Four sets of each medium were prepared; to two had been added 0.02 per cent Triton A20 prior to autoclaving. The tubes were inoculated with 0.003 or 0.000003 cc. of a 10 day old culture of H37Rv in Tween albumin medium, diluted in 0.3 cc. distilled water. These inocula corresponded approximately to 3×10^{-4} and 3×10^{-7} mg. of dry bacilli per cc. of medium. Final readings of

¹ Bovine albumin (serum fraction V) was obtained in a desiccated form from Armour Laboratories, Chicago.

² Triton A20 was generously supplied by Rohm and Haas Company, Philadelphia.

macroscopic evidence of growth, confirmed in some cases by microscopic examination, were made after 10 days' incubation at 37°C.

TABLE I
Comparative Effects of Sphingomyelins, Cerebrosides, and Serum Albumin on the Growth of Tubercle Bacilli

Test substances added to the medium			Inoculum H37Rv (mg. dry bacilli per cc. medium)			
			3×10^{-4}		3×10^{-7}	
			Basal medium	Basal medium + 0.02 per cent Triton A20	Basal medium	Basal medium + 0.02 per cent Triton A20
	source*	Final concentration per cent				
Kidney sphingomyelin	P.A.L. (1126)	0.05	5†	7†	2†	3†
		0.01	3	5	1	2
Liver	" (1127)	0.05	6	8	2	2
		0.01	4	6	2	3
Brain	" (1131)	0.05	5	7	2	3
		0.01	3	5	1	3
Lung	Thannhauser	0.05	5	7	2	3
		0.01	3	5	2	3
Cerebron	P.A.L. (928)	0.05	2	2	0	0
		0.01	3	5	2	3
Cerebron	" (932)	0.05	1	0	0	0
		0.01	2	2	0	0
Phrenosin	" (1115)	0.05	2	1	0	0
		0.01	2	1	0	0
Serum albumin		0.5	7	8	3	4
H ₂ O			2	2	0	0

* The initials P.A.L. indicate that the material was obtained from the chemical collection of the late Dr. P. A. Levene maintained at the Rockefeller Institute. The number in parentheses refers to the classification number in this collection. The sample of lung sphingomyelin was received from Dr. S. J. Thannhauser of Boston.

† The amount of growth is indicated in terms of an arbitrary ascending scale (from 0 to 8) based on gross macroscopic examination. The figure 8 corresponds to approximately 0.4 mg. dry bacilli per cc. of medium.

The results presented in Table I show that, after 10 days' incubation, growth in the absence of either serum or lipid could be detected only in the tubes having received the larger inoculum. At that time, growth was abundant in all the

albumin media irrespective of the size of the inoculum. The cerebroside preparations did not improve, and in fact decreased, the ability of the medium to support the growth of the small inocula. All preparations of sphingomyelin, on the contrary, were almost as effective as serum albumin in enhancing the yield of bacilli and in allowing the proliferation of the small inocula. Addition to the medium of the wetting agent Triton A20 improved the performance of sphingomyelin on several accounts: (a) it dispersed the phospholipid, thereby rendering the medium more limpid; (b) it increased the bacillary yield; (c) it

TABLE II

Comparative Protective Effects of Sphingomyelin and Albumin against the Toxicity of Fatty Acids

Final concentration in basal medium of			Growth 10 days after inoculation with 3×10^{-4} mg. H37Rv per cc. medium
Fatty acid	Albumin	Sphingomyelin	
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Oleic acid 0.03	0	0	0*
" " "	0	0.1	7
" " "	0.5	0	8
" " 0.01	0	0	0
" " "	0	0.1	7
" " "	0.5	0	7
Lauric acid 0.01	0	0	0
" " "	0	0.1	5
" " "	0.5	0	6
Capric acid 0.01	0	0	0
" " "	0	0.1	5
" " "	0.5	0	6
H ₂ O	0	0	3
"	0	0.1	5
"	0.5	0	6

* Symbols same as in Table I.

caused the growth to be fairly well dispersed (diffuse growth) instead of being granular as was the case in media containing either albumin or lipids without the wetting agent.

The Neutralizing Effect of Sphingomyelin on the Toxicity of Long Chain Fatty Acids.—As shown in earlier publications, serum albumin promotes the proliferation of small inocula of tubercle bacilli not necessarily by supplying nutritive factors, but rather by protecting the organisms against the toxic effect of various injurious agents (3, 4, 7). The following experiment reveals that sphingomyelin can exert a similar protective effect against the toxicity of long chain fatty acids.

The sodium soaps of caproic, lauric, or oleic acid were added to the basal medium in the concentrations indicated in Table II. Different samples of sphingomyelin were added in

amounts of 0.3 cc. of 1 per cent emulsion per 3 cc. of medium, in comparison with 0.3 cc. of 5 per cent albumin or 0.3 cc. of water. All tubes were inoculated with 0.003 cc. of H37Rv culture in Tween-albumin medium diluted in 0.3 cc. distilled water (corresponding approximately to a final inoculum of 3×10^{-4} mg. dry bacilli per cc. of medium). Macroscopic evidence of growth was read after 10 days' incubation at 37°C. The results are recorded in Table II (the data refer to only one of the preparations of sphingomyelin tested: P.A.L. 1127).

The results presented in Table II confirm earlier findings that the inhibitory effect of long chain fatty acids on the growth of tubercle bacilli can be neutralized by addition of serum albumin to the medium and establish that sphingomyelin exhibits a similar protective effect. It is of interest in this respect that addition of this phospholipid to emulsions of sodium soaps increases appreciably the apparent water solubility of the latter, suggesting the formation of a complex such as has been recognized to occur between fatty acids and albumin.

The Effect of Lignoceric Acid on the Growth of Tubercle Bacilli.—Sphingomyelin is a diamminophospholipid in which a C24 hydroxy acid (lignoceric acid) is combined in amide linkage with the base sphingosine, phosphocholine being esterified on the latter. We have not yet studied the effect of phosphocholine on bacterial growth. All samples of sphingosine tested so far have failed to enhance and indeed have caused a marked inhibition of growth; the nature of this inhibition will be considered in a subsequent publication. On the contrary, both lignoceric acid and its amide have been found to exert a beneficial effect on growth as shown in the following experiment.

Solutions of lignoceric acid and lignoceric amide in chloroform were added in graded amounts to the basal medium containing 0.02 per cent of Triton A20. Constant agitation while heating to eliminate the chloroform yielded a fairly stable emulsion of the lipids in the medium, despite their very low water solubility. The media were distributed in 4 cc. amounts in test tubes (25 mm. diameter) and autoclaved. Serum albumin was then added in final concentrations of 0.5 per cent to a duplicate set. The inoculum was 0.04 cc. or 0.004 cc. of a 10 day old culture of H37Rv diluted in 0.4 cc. distilled water (this corresponded approximately to inocula of 3×10^{-2} or 3×10^{-3} mg. dry bacilli per cc. of final medium). Macroscopic evidence of growth was recorded after 10 days' incubation at 37°C.

The results presented in Table III establish that, like sphingomyelin and oleic acid, lignoceric acid and its amide can enhance the growth of tubercle bacilli. The latter substances allow growth of small inocula and are therefore less toxic than oleic acid. This may be due to their very low solubility in water and, in the case of lignoceric amide, to the fact that the carboxyl group is masked.

On the basis of the information summarized in Tables II and III, it appears therefore that sphingomyelin exerts a favorable effect on growth by a dual mechanism. Like serum albumin, on the one hand, it protects the bacilli against certain toxic effects, in particular those of long chain fatty acids. On the other hand it constitutes a water-dispersible source of lignoceric acid which is available for metabolic utilization. In addition to their bearing on the

metabolism of tubercle bacilli, these facts may be of significance in the analysis of the factors which condition the proliferation of tubercle bacilli *in vitro*. Although sphingomyelin was first recognized in nervous tissue, it appears to be a constituent of many if not of all types of cells; and it is probable that its release from these cells at the site of infection and in caseous material would not be without effect on the growth of the bacilli.

TABLE III

Comparative Effects of Sphingomyelin, Lignoceric Acid and Amide, Sphingosine and Oleic Acid on the Growth of Tubercle Bacilli

Test substances added to the medium			Inoculum H37Rv (mg. dry bacilli per cc. of medium)			
			3×10^{-3}		3×10^{-4}	
			Basal medium	Basal medium + 0.5 per cent albumin	Basal medium	Basal medium + 0.5 per cent albumin
	Source*	Final concentration <i>per cent</i>				
Sphingomyelin	P.A.L. (1127)	0.02	8*	8*	3*	8*
Lignoceric acid	" (1076)	0.01	7	8	2	8
" "	" (1077)	"	7	8	2	8
" amide	" (1074)	0.012	7	8	3	8
Sphingosine	" (1140)	0.01	0	4	0	0
Oleic acid	"	"	0	8	0	6
" "		0.003	0	8	0	6
H ₂ O			4	6	0	4

* Symbols same as in Table I.

SUMMARY

All preparations of sphingomyelin tested, whatever the tissues from which they originated, were found to enhance the growth of tubercle bacilli *in vitro*. Cerebrosides were inactive in this respect.

Sphingomyelin promotes growth through two independent mechanisms:

(a) It neutralizes the toxicity of long chain fatty acids probably by forming with them inert complexes. This protective effect facilitates initiation of growth from small inocula.

(b) It supplies to the bacteria lignoceric acid (or its amide) which is utilized for growth. The base sphingosine, another component of sphingomyelin, does not favor and probably inhibits proliferation of tubercle bacilli.

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THE EFFECT OF WETTING AGENTS ON THE GROWTH OF TUBERCLE BACILLI

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It is possible to obtain finely dispersed growth of tubercle bacilli by adding to the media in which they are cultivated certain non-ionic wetting agents. The polyoxyethylene esters of oleic acid (Atlas G-2144), lauric acid (Atlas G-2124), sorbitan monooleate (Tween 80), sorbitan monostearate (Tween 60), sorbitan monopalmitate (Tween 40), and sorbitan monolaurate (Tween 20), have proven especially effective in this respect. In addition to their wetting effect on the cell, certain of the water-dispersible esters just mentioned are capable of enhancing the growth of many strains of tubercle bacilli, probably by supplying them with long chain fatty acids in a non-toxic form available for metabolic utilization (3). Unfortunately, the ester linkage in these wetting agents is susceptible to enzymatic hydrolysis by lipases, a fact which prohibits their use in media containing animal tissues or fluids rich in these enzymes. It is therefore desirable to find other types of wetting agents capable of promoting dispersed growth of tubercle bacilli and stable in the presence of animal tissues. The present report describes the properties of an arylalkyl polyether of phenol (Triton A20) which in some respects fulfills these requirements.

EXPERIMENTAL

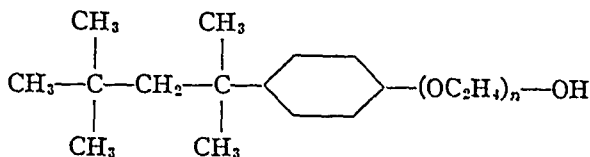
Cultures.—For the sake of brevity only experiments carried out with the human strain of tubercle bacillus H37 and with the avian strain Camden 3 will be described. Results with other strains have confirmed the findings to be presented here. Two variants of the culture H37 were used; both had been obtained originally from the Trudeau Laboratory through the generosity of Dr. W. Steenken, Jr. The virulent variant, H37Rv, was reisolated from the lung lesions of experimentally infected mice. The avirulent variant, H37Ra, was reisolated from a colony exhibiting characteristic morphology on oleic acid-albumin agar. The cultures were maintained by weekly transfers in Tween 80-albumin liquid medium and possessed the morphological characteristics of the virulent and avirulent forms, respectively (7). The avian strain Camden 3 was recently isolated in our laboratory from the liver of a tuberculous chicken and was also maintained in Tween-albumin agar. The cultures used for inoculation had been incubated for 8 to 10 days at 37°C. and contained approximately 0.35 mg. bacilli (dry weight) per cc. of medium.

Media.—The basal medium has been described in preceding reports (6); bovine serum albumin was added aseptically (in a final concentration of 0.5 per cent) after autoclaving of the medium, with or without 1.5 per cent agar. The wetting agents were added prior to autoclaving.

The sphingomyelin used was sample 1127 of the collection of the late Dr. P. A. Levene (4). It was added to the medium in the form of a 1 per cent solution in distilled water, sterilized by three consecutive heatings at 100°C.

The nature and properties of the polyethylene esters of the Tween type have been described in earlier publications (3, 5).

Triton A20 is described by the manufacturer as an arylalkyl polyether of phenol. The related compound, Triton N100, has the following general formula.



The chemical structure of Triton A20 is slightly different from that of Triton N100 and has not been published. Although both these products are related non-ionic wetting agents, Triton A20 is much less toxic than Triton N100 for tubercle bacilli and for experimental animals. For this reason, it has been selected for further study.

Triton A20 is miscible in all proportions with water and can be handled like Tween 80 in the preparation of liquid and agar media; as in the case of Tween 80, its aqueous solutions are cloudy above 80–90°C. but become limpid again at lower temperatures. Triton A20 is distributed by the manufacturer as a 20 per cent solution in water. The concentrations reported in the present publication are corrected in terms of the original material.

The media were distributed in 4 cc. amounts in Pyrex test tubes, 25 mm. in diameter. As described elsewhere, aluminum caps were used instead of cotton plugs during autoclaving and incubation (5).

Comparative Effects of Triton A20 and Tween 80 on the Growth of Tubercle Bacilli.—All cultures of tubercle bacilli so far tested are capable of giving macroscopical evidence of growth within 10 to 15 days in the basal medium to which has been added bovine serum albumin, even when the inoculum contains only a few living cells; growth in this plain albumin medium always consists of large clumps of bacilli. Tween 80 in concentration of 0.02 to 0.2 per cent increases the amount of growth and changes its character from granular to diffuse. The effect of Triton A20 was studied in the following experiment.

Tween 80 and Triton A20 were added to the basal medium in final concentrations ranging from 0.003 to 0.3 per cent as indicated in Table I. Each test medium was inoculated with 0.003 or 0.0003 cc. of a 10 day old culture in Tween albumin medium of H37Rv or H37Ra diluted in 0.3 cc. of 5 per cent bovine serum albumin. This corresponded to an inoculum of approximately 3×10^{-4} and 3×10^{-5} mg. of bacilli (dry weight) per cc. of test medium. Macroscopical evidence of growth, checked in certain cases by microscopic examination, was read after 14 days' incubation at 37°C., and is recorded in Table I.

The results presented in Table I reveal that a definite dispersing effect on the growth of virulent and avirulent variants H37Rv and H37Ra can be detected with concentrations of Tween 80 or of Triton A20 as low as 0.01 per cent. However, the dispersing effect of Tween 80 is more complete than that of the latter wetting agent. Even with the highest concentrations of Triton A20 used, the cultures of H37Rv exhibited on careful macroscopic examination a finely granular growth which was shown by microscopic study to consist of long strands of bacilli.

The two wetting agents differ also in their effect on the yield of bacilli. Addition of Tween 80 to the medium brings about a definite enhancement of growth, probably caused by the oleic acid in the Tween molecule.¹ Increase in growth does not occur with Triton A20 which apparently is not utilized by tubercle bacilli.

Finally it is obvious that whereas Tween 80 enhances to a similar degree the growth of both the H37Rv and H37Ra variants, Triton A20 behaves very differently toward the two cultures as it inhibits the growth of the latter but

TABLE I
The Effect of Tween 80 and Triton A20 on the Growth of Tubercle Bacilli

Wetting agent added to the medium		Inoculum (mg. dry bacilli per cc. of medium)			
		3×10^{-4}		3×10^{-5}	
		H37Rv	H37Ra	H37Rv	H37Ra
	Final concentration				
	<i>per cent</i>				
Tween 80	0.03	8*f.d.†	8 f.d.	6 f.d.	6 f.d.
" "	0.01	8 f.d.	8 f.d.	6 f.d.	6 f.d.
" "	0.003	6 g.	6 g.	5 g.	4 g.
Triton A20	0.3	4 d.	0	3 d.	0
" "	0.1	5 d.	2 d	4 d.	0
" "	0.03	5 d.	5 f.d.	4 d.	2 d
" "	0.01	5 d.	5 f.d.	4 d.	3 d
" "	0.003	5 g.	5 g.	3 g.	3 g.
H ₂ O		5 g.	5 g.	3 g	3 g.

* The amount of growth is recorded in terms of an arbitrary scale from 0 (no growth) to 8 (growth corresponding to approximately 0.4 mg. dry weight of bacilli per cc. of medium).

† f.d. indicates that growth was finely dispersed, exhibiting no large clumps on microscopic examination. d. indicates that the growth was dispersed but consisted of clumps readily seen with a hand lens. g. indicates granular growth consisting of large clumps or flakes.

not that of the former. It should be emphasized at this time that the toxic effect on H37Ra reported in Table I has also been observed with a number of other avirulent variants of mammalian strains of tubercle bacilli whereas none of the variants appears to be unfavorably affected by Triton A20 in concentrations of 0.05 per cent or less.

¹ It has been shown elsewhere that under certain conditions, Tween 80 can exhibit toxicity owing to its contamination with free fatty acid; the toxicity can be overcome by addition of adequate amount of serum albumin to the medium (1-3). Furthermore, we have repeatedly observed that samples of Tween which had proved entirely non-toxic when first used, develop some 2 months after the container has been opened a type of toxicity which seems to be independent of the presence of fatty acids. It is essential therefore to use only selected samples of Tween if conclusions are to be drawn concerning the effect of polyoxyethylene derivatives of sorbitan monooleate on the growth of tubercle bacilli.

The Growth-Dispersing Effect of Tween 80 and Triton A20 in Serum Media.—It has been shown in preceding publications that incubation of Tween 80 with fraction V of plasma (serum albumin) results in the liberation of free fatty acid as a result of enzymatic hydrolysis of the ester by the lipase contaminating the albumin preparation (1, 2). In experiments previously reported, lipase activity had been minimized by heating the albumin for 30 minutes at 56°C. prior to addition to the medium. On the other hand, Tween 80 is rapidly hydrolyzed in the presence of tissues or tissue fluids and there is no convenient technique for abolishing the marked lipolytic activity of these materials. As there is no

TABLE II

The Influence of Blood Serum on the Growth-Dispersing Effect of Wetting Agents

Wetting agent added to the medium		Serum		Growth 14 days after inoculation with H37Rv (3×10^{-4} mg. dry weight per cc. medium)
	Final concentration	Mouse	Ox	
	<i>per cent</i>	<i>cc.</i>	<i>cc.</i>	
Tween 80	0.05	0	0	8 f.d.*
" "	"	0.5	0	8 g.
" "	"	0	0.5	8 g.
G 2144	"	0	0	8 f.d.
" "	"	0.5	0	8 g.
" "	"	0	0.5	8 g.
Triton A20	"	0	0	5 d.
" "	"	0.5	0	8 d.
" "	"	0	0.5	8 d.
H ₂ O		0	0	5 g.
"		0.5	0	8 g.
"		0	0.5	8 g.

* Symbols as in Table I.

enzyme of animal tissues known to be capable of attacking the ether linkages of the Triton A20 molecule, it was of interest to compare Triton A20 with two water-dispersible esters of oleic acid (G 2144 and Tween 80) with respect to their ability to disperse growth of tubercle bacilli in media with or without added serum.

The wetting agents were added to the basal medium in a final concentration of 0.05 per cent. Bovine or mouse serum sterilized by filtration through porcelain candles was added in amount of 0.3 cc. per 3 cc. of medium. The tubes containing the test media were inoculated with 0.003 cc. of 10 day old culture of H37Rv diluted in 0.3 cc. of 5 per cent bovine albumin. It is known from earlier studies that this amount of albumin is sufficient to overcome the toxicity of the free fatty acid which might be released by enzymatic lipolysis of Tween 80 or G 2144. Macroscopic evidence of growth confirmed by microscopic examination was read after 14 days' incubation at 37°C. (Table II).

The results presented in Table II confirm the fact that the dispersing effect of Triton A20 on the growth of tubercle bacilli in albumin medium without serum is less complete than that of the water-dispersible esters Tween 80 or G 2144. On the other hand it is seen that in the presence of whole serum these esters completely lose their dispersing activity whereas that of Triton A20 remains unimpaired. In view of the complexity of serum and of the many unknown interactions which certainly occur between its constituents, the tubercle bacilli, and the wetting agents, it is not possible to obtain convincing evidence concerning the factors which determine the dispersed or granular

TABLE III

The Effect of Triton A20 on the Initiation and Yield of Growth of Tubercle Bacilli

Added to the basal medium (final concentration)					Growth 14 days after inoculation with (mg. cc/cc. medium)						
Tween 80	Triton A20	Oleic acid	Sphingo- myelin	Albu- min	H37Rv			H37Ra			Avian
per cent	per cent	per cent	per cent	per cent	3×10^{-4}	3×10^{-4}	3×10^{-4}	3×10^{-4}	3×10^{-4}	3×10^{-4}	3×10^{-4}
0	0	0	0	0	3 g.*	1 g.	0	2 g.	1 g.	0	1 f.d.
0	0	0	0	0.5	6 g.	4 g.	2 g.	7 g.	5 g.	1 g.	1 f.d.
0.05	0	0	0	0	3 f.d.	0	0	3 f.d.	0	0	5 f.d.
"	0	0	0	0.5	8 f.d.	5 d.	3 f.d.	7 f.d.	4 f.d.	1 d.	8 f.d.
0	0.05	0	0	0	3 d.	2 d.	0	1 d.	0	0	1 f.d.
0	"	0.02	0	0	0	0	0	0	0	0	0
0	"	0	0	0.5	5 d.	4 d.	3 d.	3 d.	1 d.	0	1 f.d.
0	"	0.02	0	"	8 d.	6 d.	3 d.	2 d.	0	0	6 f.d.
0	"	0	0.05	0	8 d.	5 d.	2 g.	6 d.	2 g.	1 g.	5 f.d.
0	"	0	"	0.5	8 d.	6 d.	4 d.	7 d.	5 d.	4 d.	8 f.d.
0	0	0	"	0	4 g.	3 g.	2 g.	4 g.	3 g.	2 g.	5 f.d.
0	0	0	"	0.5	7 g.	4 g.	3 g.	7 g.	4 g.	3 g.	7 f.d.

* Symbols as in Table I.

character of the bacterial growth. Nevertheless, the facts observed are consistent with the hypothesis that the water-dispersible esters Tween 80 and G 2144 are destroyed by the serum lipase and thereby lose their wetting properties, whereas Triton A20 remains unaffected under the same conditions.

The Effect of Triton A20 on the Initiation and Yield of Growth of Tubercle Bacilli.—Although Triton A20 can disperse the growth of tubercle bacilli it never increases significantly the yield of growth. However enhancement of growth in its presence can occur when long chain fatty acids, or sphingomyelin, are added to the medium.

Tween 80, Triton A20, oleic acid, sphingomyelin, and albumin were added to the basal medium as indicated in Table III. The media were inoculated with 0.003, 0.0003, or 0.00003 cc. of a 10 day old culture diluted in 0.5 cc. distilled water; these inocula corre-

sponded to approximately 3×10^{-4} , 3×10^{-6} , 3×10^{-8} mg. bacilli (dry weight) per cc. of medium. The amount and character of the bacterial growth were recorded after 14 days' incubation at 37°C.

The results presented in Table III illustrate again the striking differences between the wetting agents Tween 80 and Triton A20 with reference to their effects on the growth of tubercle bacilli. They confirm the finding of the preceding experiment that, in contrast with Tween 80, Triton A20 added to the basal medium with or without albumin does not increase significantly the amount of growth, although it has a definite dispersing effect on it.

Failure to enhance growth is particularly well demonstrated in the case of the avian strain Camden 3, a fact in agreement with earlier observations that all avian strains tested give only very limited growth in the absence of long chain fatty acids (3). However, addition of oleic acid to the albumin medium containing A20 definitely enhances growth which remains dispersed despite the presence of the fatty acid. The growth-promoting effect of sphingomyelin first demonstrated in the preceding communication (4) is here confirmed. It is shown moreover that the beneficial effect of sphingomyelin, rendered even more evident in the presence of Triton A20 which increases the solubility of this phospholipid, is expressed not only in terms of greater density of the culture at the end of the incubation period, but also by the fact that it allows the growth of minute inocula even in the absence of serum albumin (3).

Comparison of the results obtained with the virulent (H37Rv) and avirulent (H37Ra) variants illustrates again the selective inhibitory effect of Triton A20 on the growth of the latter culture. It is clear however, that the inhibitory effect of the wetting agent can be corrected by the addition of sphingomyelin to the medium, even in protein-free media.

Comparative Effects of Tween 80 and Triton A20 on the Morphological Characteristics of Cultures H37Rv and H37Ra.—Unlike Tween 80, Triton A20 can inhibit the growth of the avirulent variants of mammalian tubercle bacilli in concentrations which exert no detectable toxic effects on the virulent forms (Tables I and III). This difference in antibacterial activity between the two types of wetting agents is paralleled by differences in their effect on the morphological aspects of the cultures growing in their presence. Only a brief statement of these findings will be presented here as the relation of morphological characteristics to virulence is to be treated more extensively in a subsequent communication.

It will be recalled that, in the absence of a wetting agent, the virulent organisms exhibit a marked tendency to adhere to one another in the direction of their long axis; this tendency results in the formation of strands of bacilli, which can be very long and at times extend over several microscopic fields, and which are several cells in thickness. In contrast to this serpentine pattern of growth, the avirulent forms exhibit either random growth or perhaps a rosette

arrangement of the cells (7). Attenuated strains, which possess, like the BCG culture in use in our laboratory, a slight degree of "invasiveness" also exhibit intermediate morphological characteristics. On the other hand, in media containing 0.1 per cent Tween 80, cultures of the virulent organisms and of the attenuated forms grow without producing the cords characteristic of growth in the plain albumin medium, and cannot be differentiated from the totally avirulent variants. A different morphological picture is obtained in media containing the same concentration of Triton A20 (0.1 per cent). Although growth of the virulent variants in Triton A20 liquid media appears fairly disperse, and their colonies in Triton A20-albumin agar are much less rugose than those developing on plain albumin agar, microscopic examination reveals that these cultures exhibit a definite serpentine pattern of growth (cord formation). It would thus appear that Triton A20 prevents the formation of the amorphous large clumps which correspond to the granular mode of growth of tubercle bacilli in general, but does not interfere with the tendency of the virulent cells to orient themselves in the typical "cords." These observations suggest that two independent factors contribute to the morphology of cultures of tubercle bacilli: one, common to both virulent and avirulent forms, is overcome by both Tween 80 and Triton A20, the other, peculiar to the virulent variants, is affected only by Tween 80. It is also possible, however, that these differences are not of a qualitative nature but may be due to the possession by the virulent variants of larger amounts of a certain hydrophobic substance, much less abundant in the avirulent forms, and which is more readily wetted by the water-dispersible esters than by Triton A20.

Whatever the ultimate significance of these findings for the understanding of the nature of the differences in cellular structure between the virulent and avirulent variants, it appears that the wetting agents Tween 80 and Triton A20 may lend themselves to the development of selective media useful for the separation of the two types of variants and for the analysis of bacterial variation and its relation to virulence.

SUMMARY

Tween 80 and Triton A20 are two water-dispersible, non-ionic, surface-active agents which favor dispersed growth of tubercle bacilli in aqueous media probably by wetting the bacterial surface.

Tween 80 is a polyoxyethylene ester of sorbitan monooleate and is liable to enzymatic hydrolysis by lipases. Triton A20 is an arylalkyl polyether of phenol which appears resistant to the known enzymes of animal tissues.

Tween 80 loses its ability to disperse cultures of tubercle bacilli in media containing serum; Triton A20 does not.

Tween 80 increases the yield of growth, probably by supplying oleic acid to the bacilli; Triton A20 does not.

In concentrations sufficient to cause dispersed growth, Tween 80 (purified by removal of unesterified fatty acid) and Triton A20 are completely innocuous for virulent tubercle bacilli. However, Triton A20 exhibits a marked toxic effect on the avirulent variants of mammalian strains; Tween 80 does not.

The two wetting agents also differ in their effects on the morphological aspects of the bacterial cultures. Whereas Triton A20 prevents the formation of large amorphous bacillary clumps, it is less effective in preventing the orientation of the virulent bacilli resulting in the formation of long bacillary strands. Tween 80 on the contrary prevents also the formation of these cords of bacilli and exerts therefore a more effective dispersing effect on cultures of virulent tubercle bacilli.

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CORRELATION OF THE LEVEL OF HEPATIC RIBOFLAVIN WITH THE APPEARANCE OF LIVER TUMORS IN RATS FED AMINOAZO DYES*

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The effect of structure on the carcinogenicity of aminoazo dyes related to 4-dimethylaminoazobenzene (1-4) and the influence of various diets on the activity of 4-dimethylaminoazobenzene (5-7) have been the subjects of extensive study. As a result experimental conditions have been defined which make it possible to obtain a wide variation in the incidence of hepatic tumors by selecting the proper carcinogen and diet. Since no extrinsic factors, such as destruction of the dye in the diets (6), appeared to be responsible for the variations in the rate of tumor formation, a search for possible metabolic changes related to carcinogenesis was begun. Most of the initial experiments with this approach have been inconclusive. Thus, rats fed 4-dimethylaminoazobenzene in protective diets usually maintain levels of free aminoazo dyes in the blood and liver which are almost as high as those found in rats on non-protective diets (8). Since most aminoazo dyes, irrespective of their carcinogenicity, are dealkylated by the rat (1, 2, 9) the *in vivo* lability of the *N*-alkyl groups of the aminoazo dyes is not necessarily associated with the carcinogenic process. Furthermore, the over-all metabolism of 4-dimethylaminoazobenzene by rats fed various diets is similar since approximately the same levels of the same monophenyl amine metabolites are excreted in the urine irrespective of the tumor-promoting or inhibiting nature of the diet (10). However, rats fed a protective diet high in riboflavin do metabolize 4-dimethylaminoazobenzene differently from those fed diets promoting tumor induction in at least one respect, since the livers of rats on the high riboflavin diet contain less protein-bound aminoazo dye throughout the period of carcinogenesis than precancerous livers (11).

Kensler and his coworkers (12) and Griffin and Baumann (13) have observed a decrease in the level of hepatic riboflavin in the livers of rats fed certain carcinogenic aminoazo dyes. In the present paper the levels of riboflavin,

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biotin, and vitamin B₆ were determined in the livers of rats fed 4-dimethylaminoazobenzene in 10 different protective and non-protective diets for 6 and 19 weeks; similar determinations were also made on the livers of rats fed some of the same diets without the carcinogen. In addition similar analyses were performed on the livers of rats fed the dye in diets containing either urethane or increased levels of cystine or methionine. Urethane produces lung tumors in rats (14, 15) and mice (16) and a few liver tumors in rats (14), but has been reported to exert no effect on the carcinogenicity of 4-dimethylaminoazobenzene (17). Varying activities have been assigned to extra dietary cystine and methionine (18-21). Finally, the concentration of riboflavin was determined in the livers of rats fed 4-dimethylaminoazobenzene and 4 of its 5 possible C-monomethyl derivatives at several times during a 16 week period. All the data indicate that rats with a high level of hepatic riboflavin are much less likely to develop liver tumors than animals with a lower concentration, while the levels of hepatic biotin and vitamin B₆ are not correlated with the tumor incidence.

Methods

Care of the Animals.—Male Sprague-Dawley rats, 150 to 200 gm. in weight, were used exclusively. They were housed in screen bottom cages in groups of 6 to 8, and water was available *ad libitum*. The conditions used in each of the 4 series are described separately.

The object of series 1 and 2 was a comparison of the levels of certain vitamins in the livers of rats fed 4-dimethylaminoazobenzene in diets which either stimulate or inhibit the formation of hepatic tumors. The composition of the diets for series 1 is given in Table I; weighed amounts of these rations were fed so that the average food consumption for each group was 9 gm. per rat per day. Nine rats were fed each of the 10 diets with the addition of 0.06 per cent of 4-dimethylaminoazobenzene (dissolved in the oil of the diet, or, for the low fat diet, dissolved in acetone and evaporated on the diet) as well as diets 1, 3 to 5, and 7 to 10 in the absence of the dye. At least 3 animals from each group were sacrificed after 6 and 19 weeks, and their livers were analyzed for riboflavin, biotin, and vitamin B₆. In the case of tumor-bearing livers, the non-necrotic tumors and a sample of tumor-free (gross examination) liver were analyzed separately.

The control rats (group 11) in series 2 received diet 2, Table I, while groups 12, 13, and 14 received the same diet with either 0.2 per cent urethane, 0.3 per cent *dl*-methionine, or 0.5 per cent *l*-cystine, respectively, added at the expense of glucose. All the diets were fed *ad libitum*. Of the 18 rats in each group 3 were killed for analysis after 6 weeks; the livers of these animals and of 3 rats from the same stock (zero time controls) were analyzed for riboflavin and vitamin B₆. The remaining animals were examined by laparotomy at 17 weeks, continued on the same diets without the dye for an additional 8 weeks, and then killed for a final tumor count.

In series 3 and 4 the level of hepatic riboflavin was determined in rats receiving aminoazo dyes differing greatly in their carcinogenic potency. For both series the appropriate aminoazo dyes were added to diet 3, Table I; all diets were fed *ad libitum*. Series 3 consisted of groups of 18 rats fed either 0.054 per cent of 4-dimethylaminoazobenzene or 0.058 per cent of the 4'-methyl or 3'-methyl derivatives and groups of 7 which received either 0.058 per cent of 2-methyl- or 2'-methyl-4-dimethylaminoazobenzene or the basal diet (no dye added). Three rats from the same stock were killed at the beginning of the experiment, and 2 or 3 rats were

sacrificed after 1½, 3, 6, 12, and 16 weeks. Three groups of 20 rats fed either 0.054 per cent of 4-dimethylaminoazobenzene or equimolar amounts of the 4'-methyl and 3'-methyl derivatives comprised series 4. Two or 3 animals from each group were sacrificed at 1, 2, 3½, 6, 9, 13, and 22 weeks.

Analytical Methods.—Prior to analysis the rats were killed with ethyl ether, and the livers perfused *in situ* with 10 ml. of 2 per cent sodium citrate and 30 ml. of 0.85 per cent sodium chloride. Each liver was then homogenized in water in a Waring blender, and appropriate

TABLE I
Composition of Diets†*

Constituents	Diet No.									
	1	2	3	4	5	6	7	8	9	10
Casein (water-extracted), gm.	120	120		120	120	120	120	120		120
Casein (crude), gm.			120							
Egg white (ethanol-extracted), gm.									120	
Corn oil, gm.	50	50	50	200		50	50		50	50
Hydrogenated coconut oil, gm.								50		
Glucose monohydrate, gm.	790	790	770	640	840	790	790	790	790	788
Salt mixture, gm.	40	40	40	40	40	40	40	40	40	40
Detergent (penetrant 7), gm.										2.5
Rice-bran extract (vitab), gm.			20							
Riboflavin‡, gm.	0.002	0.001	0.001	0.002	0.002	0.005	0.010	0.002	0.002	0.002

* Each diet except No. 3 also contained 2.5 mg. pyridoxine hydrochloride, 3.0 mg. thiamine hydrochloride, 7.0 mg. calcium pantothenate, and 300.0 mg. choline chloride per kilo.

† We are indebted to the following for gifts of the products noted: to the late Dr. D. F. Robertson, of Merck and Company for the crystalline B vitamins; to Dr. T. M. Godfrey, of Lever Bros. Company, for the hydrogenated coconut oil; to Mr. H. R. Barnett, of Stein, Hall, and Company, for the dried domestic egg white; and to Mr. Carl Setterstrom, of Carbide and Carbon Chemicals Corp., for the tergitol penetrant 7. The "vitab rice bran concentrate" was obtained from National Oil Products Co., Harrison, New Jersey.

‡ The dietary constituents were analyzed for riboflavin and then sufficient crystalline vitamin was added to bring the total content to the level given.

aliquots were taken for each analysis. Riboflavin was determined by the method of Conner and Straub (22) after neutralization of the acid hydrolysate with sodium acetate and incubation with papain for 20 hours. The enzyme treatment increased the values by about 10 per cent above those obtained after acid hydrolysis alone. Vitamin B₆ and biotin were assayed through the growth of *Saccharomyces carlsbergensis* and *S. cerevisiae*, respectively, according to the general method of Atkin and his associates (23).¹ The results are expressed as microgram per gram of fresh liver.

RESULTS

Effect of Dietary Variations.—In general the livers of rats fed the diets containing 4-dimethylaminoazobenzene contained only about two-thirds as much

¹ We are grateful to Mrs. H. N. Kingsley for the biotin and vitamin B₆ analyses.

riboflavin as the livers of rats on the same diets without the dye (Table II). For example, the livers of the control group (group 1) fed the dye averaged

TABLE II

*The Levels of Riboflavin, Biotin, and Vitamin B₆ in the Livers of Rats Fed Protective and Non-Protective Diets. Series I**

(3 to 6 rats per group; analyses at 19 weeks)

No.	Diet	Basal diet			Basal diet + 0.06 per cent 4-dimethylaminoazobenzene				Tumor incidence (6)
		Riboflavin	Biotin	Vitamin B ₆	Riboflavin		Biotin	Vitamin B ₆	
		μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./liver	μg./gm.	μg./gm.	per cent
1	Control (2 mg. riboflavin/kg.)	20.4 (19-21)	0.90 (0.8-1.0)	10.9 (10-11)	13.7 (11-15)	121	0.53 (0.4-0.6)	7.8 (7-8)	50-80
2	Low riboflavin (1 mg./kg.)				9.8 (9-13)	95	0.58 (0.5-0.6)	7.6 (7-8)	90-100
3	Rice-bran extract	14.7 (14-15)	0.93 (0.9-1.0)	11.5 (11-12)	9.4 (9-10)	86	0.66 (0.4-1.0)	6.4 (6-8)	90-100
4	High corn oil	19.2 (17-23)	1.0 (0.9-1.1)	9.3 (8-11)	10.7 (9-13)	115	0.56 (0.5-0.7)	6.7 (6-10)	90-100
5	Low fat	19.6 (15-23)	0.58 (0.4-0.7)	11.5 (11-12)	15.2 (13-17)	110	0.58 (0.5-0.6)	7.4 (7-8)	20
6	Medium riboflavin (5 mg./kg.)				17.9 (17-18)	181	0.51 (0.5-0.6)	6.5 (6-8)	0
7	High riboflavin (10 mg./kg.)	28.6 (25-31)	1.23 (1.1-1.5)	8.7 (8-9)	19.4 (15-26)	174	0.72 (0.4-1.3)	7.5 (6-11)	0
8	Hydrogenated coconut oil	23.5 (21-27)	1.17 (1.1-1.3)	10.9 (10-12)	15.9 (12-21)	126	0.68 (0.5-1.0)	8.5 (7-10)	0-8
9	Egg white	23.6 (21-27)	0.90 (0.8-1.0)	10.4 (9-11)	17.4 (16-21)	156	0.37 (0.2-0.5)	6.0 (5-7)	0-14
10	Detergent	24.4 (23-26)	0.62 (0.5-0.7)	10.3 (9-11)	15.4 (14-17)	160	0.25 (0.2-0.3)	5.7 (5-7)	0

* The ranges for the animals in each group are given in parentheses; they are expressed to the nearest whole number for riboflavin and vitamin B₆ and to the nearest tenth for biotin.

13.7 μg. of riboflavin per gm. as compared to 20.4 μg. per gm. for those on the same basal diet; similarly, the livers of the rats on the high riboflavin diet (group 7) averaged 19.4 and 28.6 μg. per gm. for the dye-fed and basal groups,

7.9 $\mu\text{g.}$ per gm., at 6 weeks (Table III). The livers of the control group (1 mg. of riboflavin per kilo of diet) averaged 9.3 $\mu\text{g.}$ per gm., and the tumor incidence was 75 per cent at 6 months. Fifty per cent of the rats receiving 0.3 per cent *dl*-methionine developed tumors by 6 months while only 13 per cent of those supplemented with 0.5 per cent of *L*-cystine had neoplasms at this time; the livers from these groups averaged 10.7 and 10.9 $\mu\text{g.}$ of riboflavin per gm. at 6 weeks. The total hepatic riboflavin was 121, 84, and 69 $\mu\text{g.}$ per liver for the

TABLE III

*The Levels of Riboflavin and Vitamin B₆ in the Livers of Rats Fed Protective and Non-Protective Diets. Series 2**

(3 rats per group; analyses at 6 weeks)

No.	Diet†	Riboflavin		Vitamin B ₆	Tumor incidence	
		$\mu\text{g./gm.}$	$\mu\text{g./liver}$		4 mos. per cent	6 mos. per cent
Zero time		25.8 (24-26)	345	11.8 (11-13)		
11	Control	9.3 (9-10)	69	6.8 (6-7)	28	75
12	0.2 per cent urethane	7.9 (7-8)	59	6.1 (5-7)	47	80
13	0.3 per cent <i>dl</i> -methionine	10.7 (10-12)	84	8.4 (8-9)	33	50
14	0.5 per cent <i>L</i> -cystine	10.9 (10-12)	121	6.9 (6-7)	0	13

* See footnote to Table I.

† All diets contained 1 mg. of riboflavin per kg.

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ystine-supplemented, methionine-supplemented, and control rats, respectively. As in series 1 the level of vitamin B₆ could not be correlated with the tumor incidence. Our data are in substantial agreement with earlier reports which showed that cystine inhibited tumor induction to a significant extent (18-20), while methionine had little or no effect (21). The stimulation of the rate of tumor formation by urethane observed in this experiment is small and may not be significant.

Effect of Activity of Carcinogen.—When 4-dimethylaminoazobenzene was fed the level of hepatic riboflavin dropped rapidly from 22 $\mu\text{g.}$ per gm. at the beginning of the experiment to 15.6 $\mu\text{g.}$ at 1½ weeks and 10.4 $\mu\text{g.}$ at 3 weeks (Fig. 1). The riboflavin content continued to drop more slowly for the rest of the

experiment and reached 6.6 $\mu\text{g. per gm.}$ at 16 weeks. A similar pattern was followed when each of the other aminoazo dyes was fed, but the extent of the decrease varied with the carcinogenicity of the compound. With 3'-methyl-4-dimethylaminoazobenzene, which is about twice as active as 4-dimethylaminoazobenzene, the liver riboflavin fell to 8.0 $\mu\text{g.}$ in 3 weeks; at this time the livers of rats fed 2'-methyl-4-dimethylaminoazobenzene (one-third to one-half as active as 4-dimethylaminoazobenzene) contained 11.3 $\mu\text{g. per gm.}$ Livers from rats fed 2-methyl-4-dimethylaminoazobenzene, which is inactive under our condi-

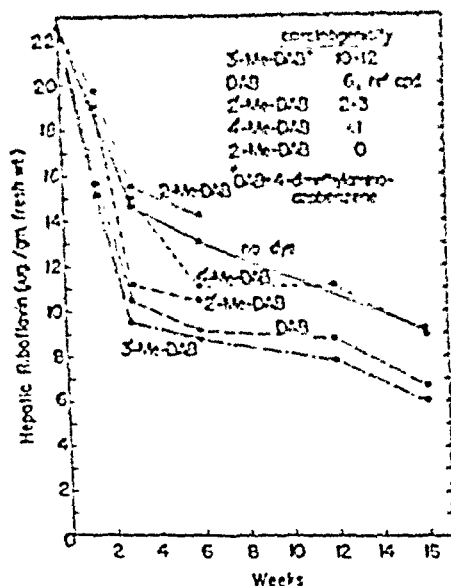


FIG. 1. The levels of riboflavin in the livers of rats fed 4-dimethylaminoozobenzene and its C-methyl derivatives.

tions, or 4'-methyl-4-dimethylaminoazobenzene, a very weak carcinogen, had approximately the same level of riboflavin as livers from animals on the basal diet. The progressive drop in the hepatic riboflavin of rats fed the basal diet is apparently a reflection of its minimal riboflavin content. Comparable results were also obtained in series 4. The riboflavin levels of the livers of rats fed 3'-methyl-4-dimethylaminoazobenzene, 4-dimethylaminoazobenzene, and 4'-methyl-4-dimethylaminoazobenzene were 12.7, 13.5, and 16.0 $\mu\text{g. per gm.}$ at 2 weeks and 8.2, 8.9, and 12.9, respectively, at 6 weeks.

DISCUSSION

The carcinogenic activity of the aminoazo dyes is related, at least in part, to the extent to which the level of hepatic riboflavin decreases when they are

fed. Thus, when various aminoazo dyes are fed in the same diet, the most rapid and greatest depression of riboflavin occurs in the livers of rats fed the most carcinogenic dyes. Similarly, the livers of rats fed 4-dimethylaminoazobenzene have lower levels of riboflavin when a non-protective diet is fed than when a protective diet is given; the levels of biotin and vitamin B₆, however, do not vary with the protective nature of the diet. The means by which the aminoazo dyes alter the riboflavin content of the liver is unknown, but alterations in the amount of intestinal synthesis, in the efficiency of absorption, or in the *in vivo* lability of riboflavin could give these results. There is little literature pertinent to the latter two possibilities, but some experiments suggest that the protective and accelerating diets could alter the amount of intestinal synthesis of riboflavin. Thus, Czaczkes and Guggenheim (25) found that rats fed a diet low in fat required less riboflavin daily to maintain their liver stores than animals receiving 10 per cent olive oil and those on a high fat diet required more riboflavin daily. From bacterial counts and riboflavin analyses on the feces they concluded that these effects were due to a greater synthesis of the vitamin by the intestinal flora in the presence of a low fat diet and a lesser synthesis with the high fat diet.

Since the level of hepatic riboflavin appears to be important in determining the resistance of the liver to 4-dimethylaminoazobenzene, the distribution of this vitamin in the liver cell has recently been investigated (26). The large granules (mitochondria) and the supernatant fluid from the homogenates of livers of rats fed the dye contained only 50 to 60 per cent as much riboflavin as the same liver fractions of rats on the basal diet, while the riboflavin content of the nuclei and small granules (microsomes) was not altered by the dye-feeding. Analyses of these fractions for total protein and nucleic acids also indicated extensive damage of the large granules by the dye.

The question still remains whether riboflavin interferes with a part of the carcinogenic process *per se* or whether it acts prior to this process. It is possible that riboflavin-containing enzymes are responsible for the destruction of the dye in the liver; thus livers with access to large amounts of the vitamin might destroy much of the dye before it has an opportunity to act as a carcinogen. For instance, Kensler (27) has found that slices of livers containing high levels of riboflavin destroy 4-dimethylaminoazobenzene faster than livers containing less of the vitamin. Preliminary data have also indicated that the protein-bound dyes are metabolized more rapidly by rats receiving high levels of dietary riboflavin (11). On the other hand, if riboflavin and 4-dimethylaminoazobenzene or their derivatives compete for a protein involved in the carcinogenic process, increased amounts of riboflavin would protect the protein and thus slow down the process of carcinogenesis. Unfortunately no data are available to support or disprove the latter idea.

SUMMARY

The livers of rats fed 10 diets previously found to inhibit or accelerate the induction of tumors by 4-dimethylaminoazobenzene were analyzed for riboflavin, biotin, and vitamin B₁₂ after 6 and 19 weeks on the diets. Those fed diets accelerating tumor induction had average hepatic riboflavin levels of 9 to 11 $\mu\text{g. per gm.}$. The livers of the control and protected rats averaged 14 and 15 to 19 $\mu\text{g. per gm.}$, respectively. The hepatic levels of vitamin B₁₂ and biotin averaged 7.0 and 0.54 $\mu\text{g. per gm.}$, respectively, and were independent of the protective nature of the diet. The livers of rats fed the same diets in the absence of 4-dimethylaminoazobenzene contained about 50 per cent more of each of these vitamins than those from dye-fed rats.

In a second series the tumor incidence and the hepatic riboflavin and vitamin B₁₂ were determined simultaneously. In this experiment urethane decreased the level of hepatic riboflavin and increased the rate of induction of tumors slightly. Methionine inhibited tumor induction to a small extent while cystine had a more pronounced retarding effect; the level of hepatic riboflavin for both groups was higher than in the controls.

The livers of rats fed the basal diet, 0.051 per cent of 4-dimethylaminoazobenzene, or equimolar amounts of its 2-, 2', 3', or 4'-methyl derivatives were analyzed for riboflavin at various times over a 16 week period. In each case the level of hepatic riboflavin decreased throughout the experimental period, but the rate and extent of the riboflavin loss were greatest with the more carcinogenic compounds.

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THE RATE OF BACTERICIDAL ACTION OF PENICILLIN IN VITRO AS A FUNCTION OF ITS CONCENTRATION, AND ITS PARADOXICALLY REDUCED ACTIVITY AT HIGH CONCENTRATIONS AGAINST CERTAIN ORGANISMS

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Penicillin has been shown to be directly and rapidly bactericidal (1-9), and not merely bacteriostatic. Whether that direct action is augmented *in vivo* by the natural defense mechanisms of the body has not yet been conclusively determined; but in any case, it is a reasonable surmise that the concentration of penicillin which is most rapidly effective against a given organism *in vitro* may also be the most effective *in vivo*. Further, if different organisms vary not only with respect to the effective concentrations of penicillin, but also in the maximal rate at which they can be killed by the drug, there may be a corresponding variation in the time for which treatment with penicillin must be continued in order to effect cure.

The present paper will describe experiments relating to the effect of the concentration of penicillin on the rate of its bactericidal action *in vitro* against a number of bacteria. As had previously been indicated by Hobby, Meyer, Dawson, and Chaffee (1, 2), Eagle and Musselman (6), and Demerec (7), the rate at which the bacteria were killed by penicillin *in vitro* was found to vary strikingly within relatively narrow zones of concentration. For every organism here studied, one could define (1) a concentration at which the net rate of multiplication was significantly reduced, (2) a somewhat higher concentration at which the organisms were killed faster than they multiplied, so that the net number of viable organisms slowly decreased, and (3) a maximally effective concentration, at which the organisms died at a maximum rate which was not further increased even by a 32,000-fold increase in penicillin concentration (*cf.* references 1, 2, 6, 7). However, the several bacterial species differed not only with respect to the magnitude of this maximally effective concentration of penicillin, but also with respect to the maximal rate at which they could be killed by the drug; and there was no necessary relationship between the two values. Finally, with many bacterial species there was a sharply defined concentration of penicillin at which it was optimally effective, and in excess of

which the rate at which the organisms were killed was paradoxically reduced rather than increased (*cf.* reference 9).

The therapeutic implications of these observations are discussed in the text.

I. METHODS AND MATERIALS

The organisms studied in the present paper included 14 strains of β -hemolytic streptococci of Lancefield groups A, B, and C; 11 strains of α -hemolytic streptococci, including 7 strains of *Streptococcus fecalis*; 7 strains of *Staphylococcus aureus*, and 2 of *Staphylococcus albus*; *Diplococcus pneumoniae*, Types I, III, VIII, XI, XII, XIV, and XXIV; and the so called Reiter strain of cultured *T. pallidum*.¹

Fifteen hour cultures grown at 30°C. were usually used as the starting material except in the case of the slow growing *T. pallidum*, for which 48 hour cultures at 37°C. on Brewer's thioglycollate medium were used. Penicillin G was added in varying concentrations to an appropriate dilution of the culture, and the mixture placed in a water bath at 37°C. Aliquot portions were taken out at intervals and (the spirochete cultures excepted) the number of surviving viable organisms was determined² by plating out serial 40-fold dilutions (0.3 cc. and 11.7 cc.) in an agar (or blood-agar) medium containing an excess of "clarase" (Takamine) in order to terminate the action of the penicillin. The number of such 40-fold dilutions was adjusted to the anticipated number of organisms. With the treponemal culture, the number of surviving and viable organisms was determined in fluid Brewer's thioglycollate medium enriched with 10 per cent rabbit serum, and containing enough agar (0.1 to 0.2 per cent) to cause the organisms to grow out in discrete colonies.

When the concentration of penicillin in the reacting mixture was in excess of 1 microgram per cc. (but less than 256), the clarase was added to the first tube of the serial subcultures to a final concentration of 0.083 per cent; all other tubes contained 0.017 per cent of clarase. When the penicillin concentration was 256 to 2048 micrograms per cc., the higher concentration of clarase was used in the first 2 tubes of the serial subcultures. Although Takamine clarase was used in most of the present experiments, a preparation of penicillinase obtained through the courtesy of the Schenley Laboratories has given equally satisfactory results.

In the tables the number of surviving and viable organisms is expressed as the percentage of the original inoculum, determined prior to the addition of the penicillin. In the figures, the proportion of survivors has been expressed as a decimal fraction, referred to the original inoculum as 1.

¹ Most of these strains were obtained through the courtesy of Dr. George F. Mirick, Dr. J. Howard Brown, and Dr. Martin Frobisher, Jr., of the Johns Hopkins Schools of Medicine and Public Health, and Dr. W. F. Verwey of the Sharp and Dohme Laboratories, Glenolden, Pennsylvania. It is a pleasure also to acknowledge the cooperation of the Squibb Institute for Medical Research, the Merck Company, Inc., and the Commercial Solvents Corporation in supplying the penicillin G used in these studies, of the Charles Pfizer Company in supplying penicillin K, and of the Lederle Laboratories in supplying the penicillin X (*cf.* page 128).

² These experiments determine the proportion of surviving organisms still viable after a given exposure to penicillin. Those bacteria still alive at the time of subculture, but which had been so damaged by penicillin that they failed to grow out in subculture, therefore appeared as dead organisms. This factor tended to make the apparent rate of bactericidal action somewhat greater than was actually the case.

A further complication was introduced by the fact that with those organisms which associated in clumps or chains, just one viable organism surviving out of a group of 10 for example, sufficed to form a colony on subculture, and the fact that the other 9 were dead or dying

II. THE RATES AT WHICH BACTERIA ARE KILLED BY PENICILLIN

A. β -Hemolytic *Streptococci*

1. *Group A (Lancefield)*.—One of 5 similar experiments with the C-203 strain of *Streptococcus pyogenes* is summarized in detail in Table I and in Fig. 1. As is there shown, as little as 0.004 microgram per cc. of penicillin G had a

TABLE I

The Rate at Which *Streptococcus pyogenes* (C-203) Is Killed at Varying Concentrations of Penicillin G

(Initial number of organisms in reacting mixture = 750,000 per cc.)

Concentration of penicillin G, micrograms per cc.												Concentration of penicillin (micrograms per cc.) which		
Time at 37°C.	0	0.004	0.006	0.003	0.012	0.016	0.024	0.032	0.048	0.064	512	reduced growth	effect of net reduction in no. of viable organisms	killed organisms at maximal rate
	Percentage* of organisms still viable after indicated time period													
hrs.	700	—	—	—	10	0.83	0.28	0.12	0.057	0.063	0.04	0.004	{	0.006-0.013-0.043-0.064
3	3,300	1,400	680	100	1	0.03	0.02	0.013	0.013	0.008	0.007			
6	15,000	1,700	21.6	4.7	0.02	0.006	0.0038	0.003	0.0026	0.002	0			
12	—	—	—	0.024	0.0012	0.0003	0.00013	0.00013	0.00013	0	0			
24	4,500	15.3	0.5	0.002	0	0	0	0	0	0	0			
Time required to kill 99.9 per cent of organisms, hrs.		>24	>24	10.5	4.8	2.5	2.2	1.9	1.6	1.5	1.4			

* Referred to original inoculum as 100.

significant effect in diminishing the net rate of growth of the organisms as compared with that in a penicillin-free control. At concentrations of 0.006

was thereby masked. This factor would tend to make the rate of bactericidal action seem less than was actually the case.

These two important sources of error have of necessity been ignored in the following discussion. The number of colonies formed on subculture after a given exposure to penicillin has been taken as the number of organisms surviving at that moment, and has been used as a measure of the rate of bactericidal action. Within a single experiment, the comparative results with a series of penicillin concentrations nevertheless remain a valid measure of their relative activity.

and 0.008 microgram per cc., the number of viable organisms at first increased over a 3 hour period, or remained stationary (growth approximately equal to rate of death), and gradually fell off thereafter. A slight increase in concentration, to 0.012 microgram per cc., had a striking effect on the rate of death.

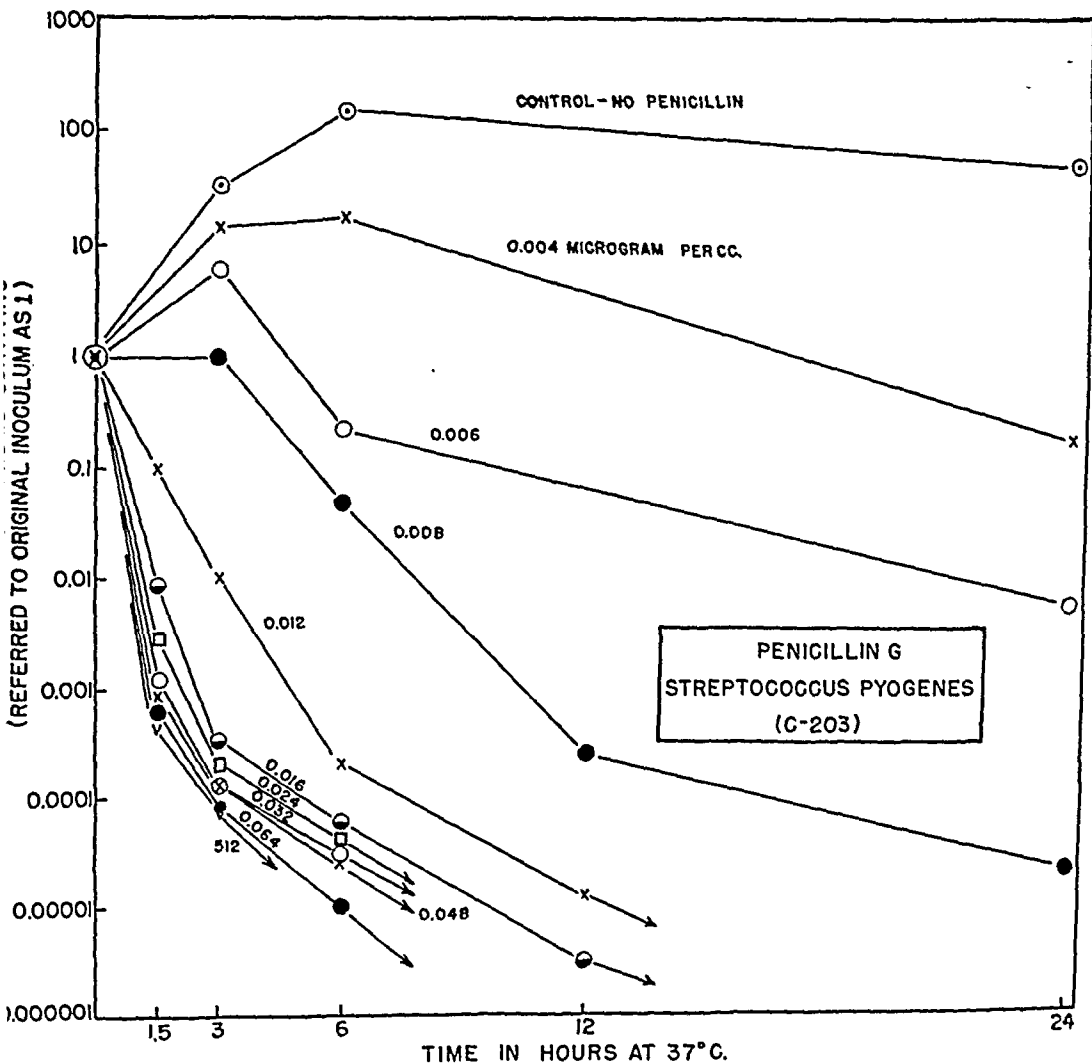


FIG. 1. The effect of the concentration of penicillin on the rate at which *Streptococcus pyogenes* (C-203) was rendered non-viable by penicillin G. (After data of Table I.)

Thus, the number of viable organisms surviving after 6 hours' exposure to 0.012 microgram per cc. was 1/5000th of the original inoculum, and was 1/240th of the number surviving in the tube containing just 33 per cent less penicillin (0.008 microgram per cc.). As the concentration of penicillin was further increased to 0.016, 0.024, and 0.032 microgram per cc., the rate of

bactericidal action also increased, but to a smaller degree with each succeeding increment in concentration. A maximum effect was obtained with a concentration of 0.064 microgram per cc., at which 99.998 per cent of the organisms were killed in the first 6 hours, and 99.94 per cent in the first 90 minutes, with no

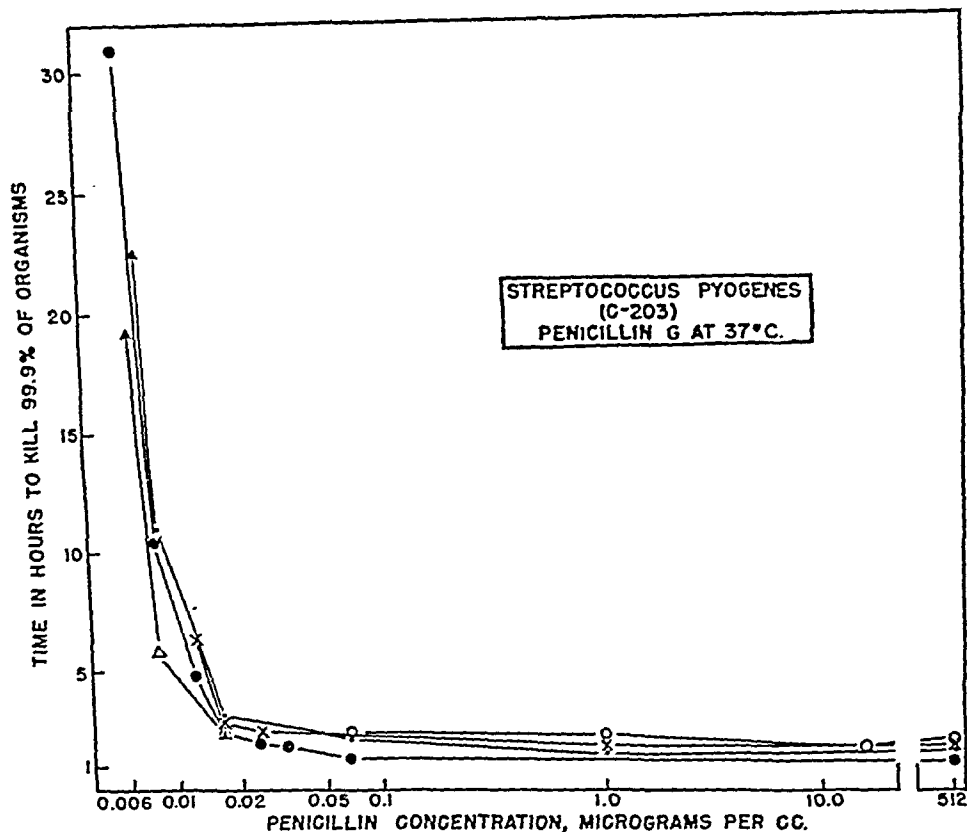


FIG. 2. The effect of the concentration of penicillin G on the time required to kill 99.9 per cent of the organisms in a suspension of *Streptococcus pyogenes* (C-203). (Summary of 5 experiments resembling that of Fig. 1 and Table I.)

indication of an initial "lag" period in the action of penicillin. Further increase in the concentration of penicillin, to as high as 512 or 2048 micrograms per cc. (32,000 times the maximally effective level of 0.064 microgram per cc.) did not further accelerate the rate at which the organisms were killed (*cf.* Hobby, Meyer, and Chaffee (1); Demerec (7)).

Five similar experiments with the same C-203 strain, all with qualitatively and quantitatively similar results, are graphically summarized in Figs. 2 and 3. In Fig. 2 the results are plotted to show the effect of the concentration on

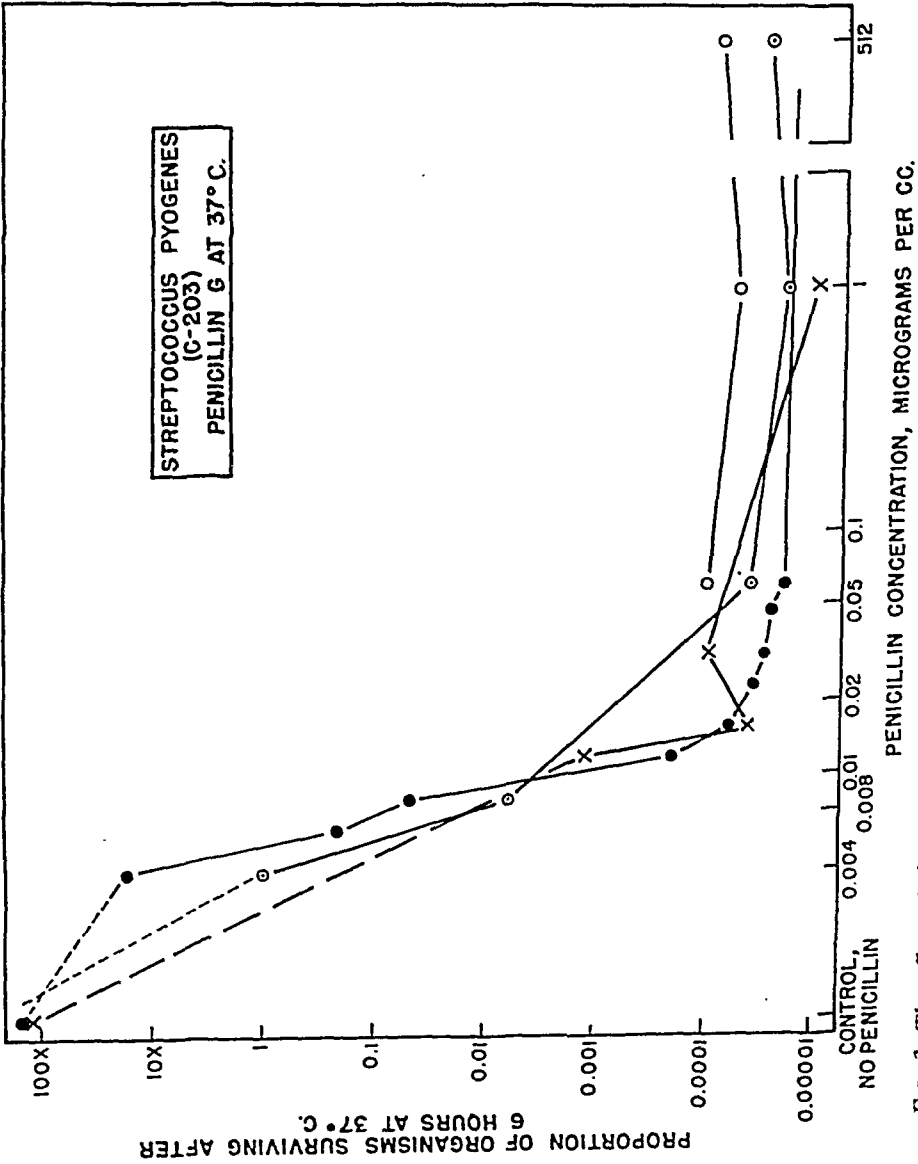


FIG. 3. The effect of the concentration of penicillin G on the proportion of organisms surviving after 6 hours at 37°C. (*Streptococcus pyogenes* (C-203)). (Summary of 4 experiments resembling that of Fig. 1 and Table I.)

the time required to kill 99.9 per cent of the organisms; and Fig. 3 shows the effect of penicillin concentration on the proportion of viable organisms after 6 hours' incubation at 37°C. In both of these figures, the graph with filled circles (—•) refers to the experiment of Table I and Fig. 1.

With this strain of streptococcus, a 15-fold difference in penicillin concentration therefore comprised the total range of bactericidal activity, from concentrations which served only to reduce the net rate of multiplication (0.004 micro-

TABLE II

The Susceptibility of 5 Strains of Group A β -Hemolytic Streptococci to Penicillin G

Strain No.	Concentration of penicillin G, micrograms per cc.							Concentrations of penicillin (micrograms per cc.) which			Concentration of penicillin G, micrograms per cc.						
	0	0.004	0.008	0.016	0.032	0.064	256	decreased net rate of multiplication	caused net decrease in no. of viable organisms	killed organisms at maximal rate	0.008	0.016	0.032	0.064	0.125	256	
	Percentage of organisms surviving after 6 hrs. at 37°C.										Time required to kill 99.9 per cent of organisms, hrs.						
F24*	9,500	3,800†	1,440‡	1.9	0.022		0.022	0.004	0.016	0.032	"	4.4	2.6	3.7	4.1	2.8‡	
M24	4,500	9,000 (P)	430	0.05	0.03	0.1	0.02	0.004-0.005	0.016	0.032	"	5.4	4.7	6	4		
M25	17,200		425	0.018	0.015		0.004	0.004-0.005	0.016	0.032	"	3.8	4			4	
M27	57,100		1,100	0.87	0.89	0.31	0.016-0.004-0.003 (?)	0.016	0.016	0.032	"	8.3	8.3	7.8		4.5 (9)‡	
C-203§	15,000	1,700	4.7	0.006	0.003	0.0026	—	0.004	0.016	0.032-0.064	10.5	2.5	1.9	1.5	—	1.4	

* This strain was killed more rapidly than the others, and the values given are the percentage of viable survivors after 3 hours, rather than 6.

† These fill-in values were obtained in a second experiment.

‡ Experiment of Table I.

gram per cc.), to concentrations at which the organisms were killed at a maximal rate (0.064 microgram per cc.).

Four other group A strains gave results qualitatively similar to those obtained with the C-203 strain described above: concentrations of 0.008 microgram per cc. regularly reduced the net rate of multiplication; a maximal rate of bactericidal action was effected by concentrations of 0.016 to 0.032 microgram per cc.; and that rate was not affected by further increase in the concentration of penicillin up to as much as 256 micrograms per cc. (cf. Table II).

2. *Group B β -Hemolytic Streptococci.*—A single experiment with a group B β -hemolytic streptococcus is given in detail in Table III, and another experiment with the same strain is shown graphically in Fig. 4. Similar experiments

with 4 different strains are summarized in Table IV. As there shown, with all 4 strains, concentrations of 0.016 microgram per cc. sufficed to reduce the rate of multiplication, and concentrations of 0.032 to 0.064 microgram per cc. effected a net bactericidal action which was maximal at concentrations of 0.064 to 0.25 microgram per cc.

With further increase in the concentration of penicillin, however, the rate of death did not stabilize at that maximum as in the case of the group A organisms previously studied. Instead, the maximal effect was observed only within a narrow optimal range of penicillin concentration. With further increase, the organisms were killed more slowly, and progressively more slowly the higher the concentration of penicillin. Thus, with each of the 4 strains there were

TABLE III

A Paradoxical Zonal Effect in the Susceptibility of a Group B β -Hemolytic Streptococcus (Strain F20) to Penicillin G

Time	Concentration of penicillin, micrograms per cc.				
	0	0.032	0.064	0.128	256
	Percentage of organisms still viable (inoculum = 100)				
hrs.					
3	2,600	42	4	1.8	55
6	16,600	32	0.056	0.079	15
12	35,600	52	0.0048	0.016	4
24	47,700	87	0	0.0004	0.08
Time required to kill 99.9 per cent of organisms, hrs.....		>	5.6	5.8	23

from 10 to 200 times as many survivors after 6 hours' exposure to 256 micrograms of penicillin per cc. as there were after similar exposure to the optimal concentration of 0.064 to 0.25 microgram per cc. (*cf.* Table IV). This zonal effect, which is illustrated for the F20 strain in Fig. 4, was also apparent when the results were expressed in terms of the time required to sterilize the culture. At the optimal concentration of 0.1 microgram per cc., it required an average of 6, 7, 3.5, and 6 hours to kill 99.9 per cent of strains M22, F20, F21, and F22, respectively. The corresponding times required at concentrations of 1 microgram per cc. were 16, 21, 8, and 11 hours. This retarded activity of penicillin in high concentration was noted by Garrod (23), while studying the rate at which staphylococci were killed by the drug. However, he ascribed this phenomenon to the presence of impurities in the penicillin, an interpretation which is not consistent with the data here presented (*cf.* page 128).

3. *Group C.*—Of the 5 strains of group C streptococci listed in Table V, 2 (M20 and M26) were killed at a maximal rate by relatively low concentrations

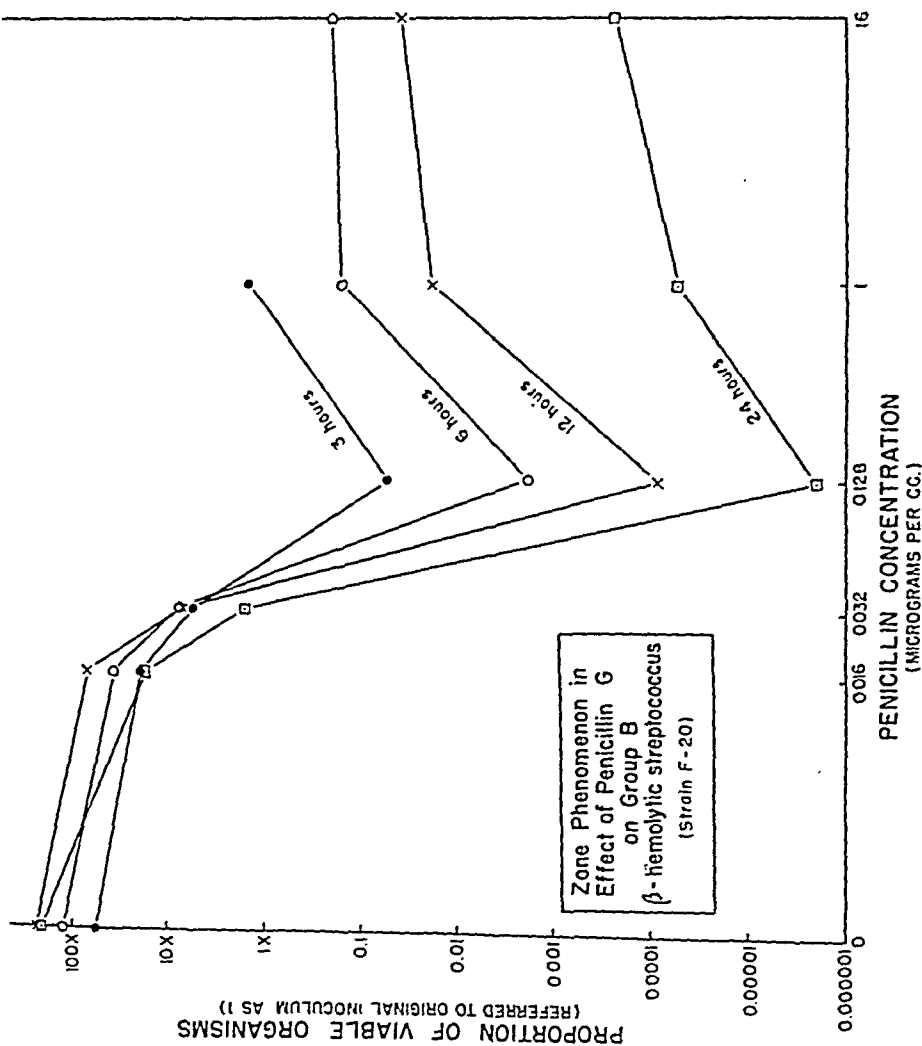


FIG. 4. The effect of the concentration of penicillin on the proportion of surviving viable organisms after 3, 6, 12, and 24 hours (experiment with a group B β -hemolytic streptococcus, illustrating the paradoxically decreased rate of death at higher concentrations of penicillin).

of penicillin (0.016 to 0.032 microgram per cc.), and that maximal rate was unaffected even by an 8,000-fold increase in the concentration of the drug. The other 3 strains, however, showed a pronounced zonal susceptibility to penicillin, similar to that described for the group B organisms in the preceding

TABLE IV

A Paradoxical Zonal Effect in the Susceptibility of Group B β -Hemolytic Streptococci to Penicillin (Illustrative Experiments with 4 Strains)

Strain No.	Concentration of penicillin (micrograms per cc.) which			Zone phenomenon at higher concentrations	Percentage of organisms surviving after 6 hrs. exposure to		Time required to kill 99.9 per cent of organisms at	
	reduces growth	effects net reduction in no. of viable organisms	kills organisms at maximal rate		optimal concentration of penicillin	256 micrograms per cc.	optimal concentration of penicillin	256 micrograms per cc.
							hrs.	hrs.
M22	0.016	0.064	0.128-0.25	+	0.04	6.6	6-7	16
F20	0.016	0.032-0.064	0.064	+	0.08	15	6-7	21-23
F21	<0.016	0.064	0.064-0.128	+	0.018	1.1	3.5	8.5
F22	0.016	0.064	0.064-0.128	+	0.11	1.26	6	12

TABLE V

The Susceptibility of 5 Strains of Group C β -Hemolytic Streptococci to Penicillin G

Strain No.	Concentration of penicillin (micrograms per cc.) which			Zone phenomenon at higher concentrations	Percentage of organisms surviving after 6 hrs. exposure to		Time required to kill 99.9 per cent of organisms at	
	reduces growth	effects net reduction in no. of viable organisms	kills organisms at maximal rate		optimal concentration of penicillin	256 micrograms per cc.	optimal concentration of penicillin	256 micrograms per cc.
							hrs.	hrs.
M20	0.004	0.016	0.016-0.032	\pm	0.03	0.39	3.9	>9
M26	0.004	0.016	0.032	0	0.022	0.02	3.8	4.7
F23	0.004	0.016	0.032	+	0.017	1.8	4	13.2
M23	0.004	0.016-0.032	0.032	+	0.71	15	9.1, 8	14, 21
M31*	1	4	4-8	+	1.7	74	17	80

* Identification as group C β -hemolytic streptococcus questionable.

section. These 3 strains were killed most rapidly within a narrow range of concentrations, which centered at 0.032 microgram per cc. for strains F23 and M23, and 4 to 8 micrograms per cc. for strain M31. (The identification of the latter discrepant strain as a group C organism is, however, questionable; cf. footnote to Table V.) A relatively slight increase beyond those optimally effective levels markedly retarded the rate of death. This is shown in Fig. 5, which relates to strain F23 of Table V. The zone phenomenon was further

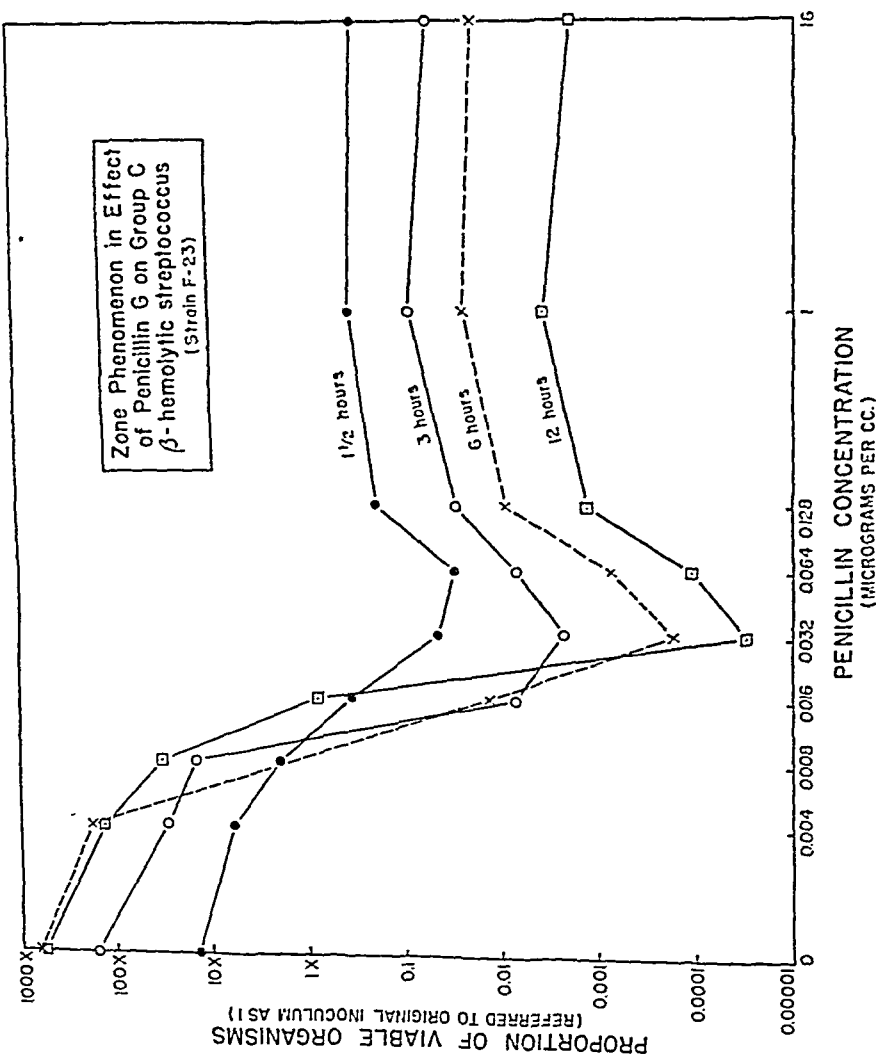


Fig. 5. The effect of the concentration of penicillin on the proportion of surviving viable organisms after 3, 6, 12, and 24 hours (experiment with a group C β -hemolytic streptococcus, illustrating the paradoxically decreased rate of death at higher concentrations of penicillin).

evidenced in the times required to kill 99.9 per cent of the organisms, as summarized in the last section of Table V.

B. α-Hemolytic Streptococci

1. *Streptococcus fecalis*.—One of 5 experiments with a single zone-sensitive strain of *Streptococcus fecalis* is summarized in Table VI. Of 7 strains of *Streptococcus fecalis* similarly tested, 5 showed the same zonal phenomenon in the action of penicillin, and were remarkably uniform in their susceptibility to the drug. As is indicated in Fig. 6, the smallest concentration with a net bactericidal action was 3 micrograms per cc.; all 5 strains were most rapidly

TABLE VI

The Effect of the Concentration of Penicillin G on the Rate of Its Direct Bactericidal Action on Streptococcus fecalis

(Illustrating one of 5 similar experiments with the same strain: original inoculum = 667,000 per cc.)

Time	Concentration of penicillin G, (micrograms per cc.)										
	0	2	3	4	6	8	12	16	32	512	2,048
	Percentage of organisms still viable after indicated time period										
hrs.											
1½	1,950	67	4.7	3.5	2.2	3.24	7.2	10.4	20	60	65
3	7,900	49	0.36	0.18	0.28	0.44	1.32	2.0	5.6	48	60
6	29,400	26	0.046	0.037	0.031	0.049	0.16	0.14	1.1	13	29
12	47,400	700	0.026	0.007	0.0075	0.009	0.044	0.054	0.36	5.6	6.4
24	64,000	34,000	3,200	>100	0.005	0.0015	0.0075	0.0025	0.006	0.56	1.12
Time required to kill 99.9 per cent of organisms, hrs.		∞	5.4	4.1	4.4	5	8	8.1	16	33±	40

killed at concentrations of 4 to 6 micrograms per cc.; and the higher the concentrations in excess of that level to which the organisms were exposed, the more slowly they were killed. Thus, after 6 hours' exposure to concentrations of 64 micrograms per cc., there were, on the average, 20 times as many survivors as there were at concentrations of 4 to 6 micrograms per cc.; and at 512 micrograms per cc. the ratio was more than 400-fold. At 4, 16, 64, and 512 micrograms per cc., it required an average of 5.1, 13, 27, and 40 hours, respectively, to kill 99.9 per cent of the organisms, a progressive decrease in the rate of bactericidal action of penicillin as its concentration was increased.

The remaining 2 strains did not show the zone phenomenon under discussion. Like the other 5 strains (*cf.* top curves of Fig. 6), they were killed at a maximal rate at a concentration of 4 to 6 micrograms per cc.; but as with group A β-hemolytic streptococci, that maximal rate was not thereafter affected by a

128-fold increase in dosage. It is of interest that these 2 "non-zonal" strains were killed only slowly by penicillin even at optimal concentrations. More than 48 hours were required to kill 99.9 per cent of the organisms, as contrasted with an average of 5 hours for the "zone-sensitive" organisms.

2. *Other Strains of α -Hemolytic Streptococci.*—Five other strains of α -hemolytic organisms were similarly studied (Table VII). In all, growth was significantly inhibited at concentrations of 0.016 to 0.032 microgram per cc.; with

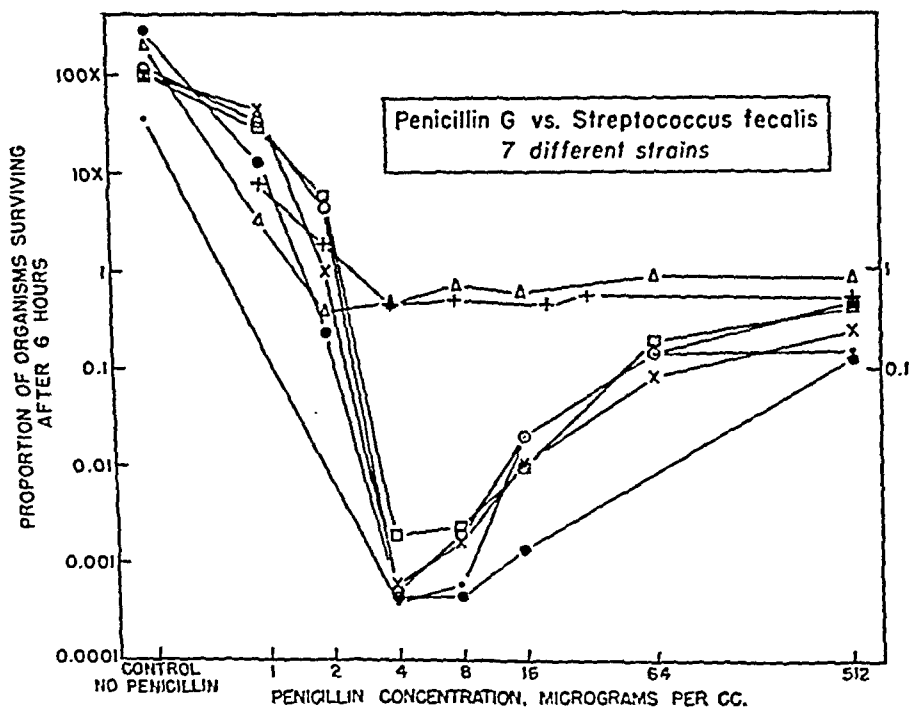


FIG. 6. The effect of the concentration of penicillin on the proportion of organisms surviving after 6 hours at 37°C. (7 strains of *Streptococcus fecalis*, of which 5 show the zone phenomenon).

all, twice that concentration was clearly bactericidal, and concentrations of 0.064 to 0.0128 microgram per cc. were maximally effective. With 3 of the 5 strains, further increase in penicillin concentration beyond the optimally effective level caused the typical retardation of penicillin action, graphically shown in Fig. 7.

As in the case of *Streptococcus fecalis* (see above) there was no necessary correlation between the concentration of penicillin necessary to kill a given strain, and the absolute rate of that bactericidal action. Thus, there was only a 2- to 4-fold difference in the optimum concentration of penicillin for strains F25 and M29 at the bottom of Table VII. At those optimal concentrations,

TABLE VII

The Susceptibility of 5 Strains of α -Hemolytic Streptococci to Penicillin G

Strain No.	Concentration of penicillin (micrograms per cc.)* which			Zone phenomenon at higher concentrations	Percentage of organisms surviving after 6 hrs.† exposure to		Time required to kill 99.9 per cent of organisms at	
	reduces growth	effects net reduction in no. of viable organisms	kills organisms at maximal rate		optimal concentration of penicillin	256 micrograms per cc.	optimal concentration of penicillin	256 micrograms per cc.
							hrs.	hrs.
F26	0.016-0.032	0.032-0.064	0.064	+	1.12	30.6	6.5	34
F27	0.016-0.032	0.032-0.064	0.064	+	0.12	10.5	7.0	26
M28	0.016-0.032	0.032-0.064	0.128	+	0.67	40	10	45
F25	0.016	0.032	0.064	0	0.029†	0.05†	2.8	2.4
M29	0.016-0.032	0.064-0.128	0.128	0	15.8	20.2	25	29

* Based on a number of experiments with each strain, each similar to that of Tables I and III.

† This strain was killed more rapidly than the others, and the values given are the percentage of viable survivors after 3 hours, rather than 6.

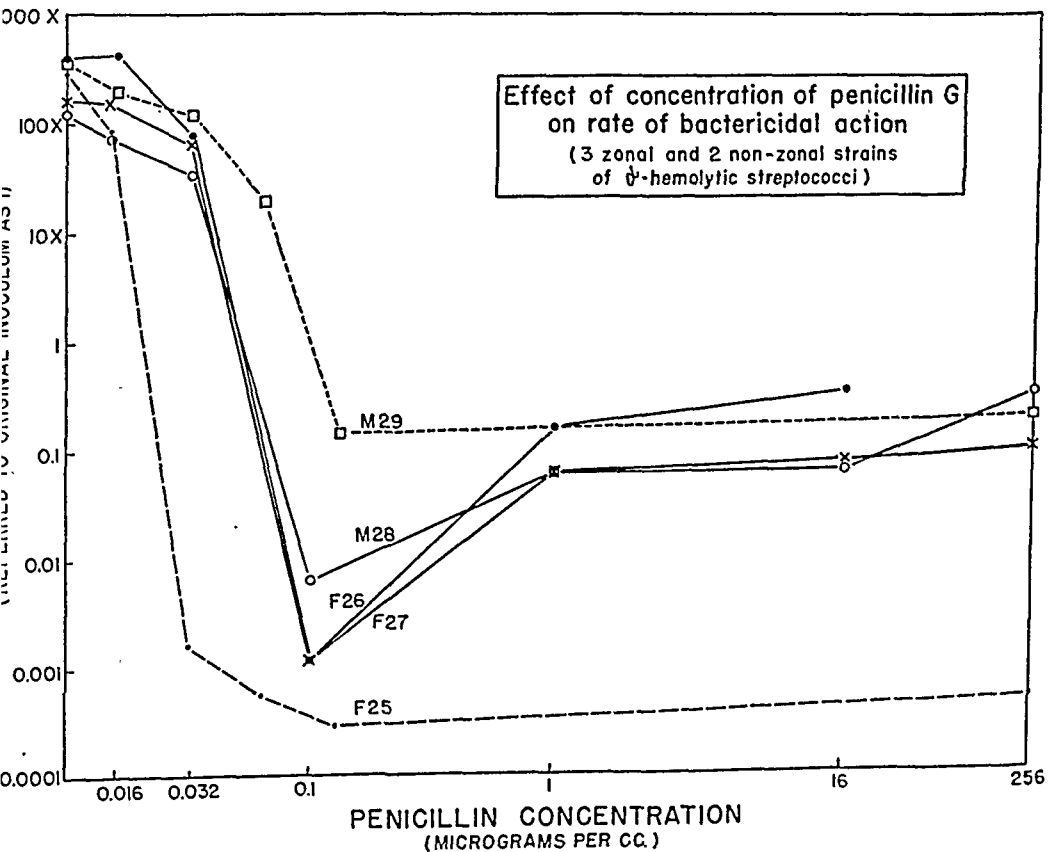


FIG. 7. The effect of the concentration of penicillin on the proportion of surviving viable organisms after 6 hours at 37°C. (5 strains of α -hemolytic streptococci, 3 of which showed a zone phenomenon; i.e., decreased rate of death at higher concentrations of penicillin).

however, the proportion of survivors after 6 hours differed more than 1500-fold (<0.01 and 16 per cent, respectively), and the times required to kill 99.9 per cent of the organisms differed 10-fold (2.4 and 25 hours, respectively).

C. Diplococcus pneumoniae

One of 5 similar experiments with a Type I strain of pneumococcus is summarized in Table VIII. A concentration of 0.016 microgram per cc. significantly reduced the rate of multiplication; the smallest concentration which

TABLE VIII
The Effect of the Concentration of Penicillin G on the Rate of Its Pneumococcal Activity in Vitro

(Illustrating one of 5 experiments with a Type I strain of *Diplococcus pneumoniae*: original inoculum = 1.08×10^8 per cc.)

Time	Concentration of penicillin G, micrograms per cc.									
	0	0.012	0.016	0.024	0.032	0.048	0.064	1	16	512
Percentage* viable after indicated time period										
hrs.										
1½	220	93	120	97	61	9.5	4	5.5	13	24
3	3,300	400	200	1.2	0.25	0.16	0.1	0.09	0.8	3
6	6,900	1,600	320	1.1	0.042	0.036	0.054	0.04	0.05	0.075
12	12,000	620	—	—	—	0.0022	0.005	0.008	0.005	—
24	—	—	840	0.72	0.125	0	0.0006	—	—	—
Time re- quired to kill 99.9 per cent of organ- isms, hrs.	∞	∞	∞	>24	4.5	3.7	3	3	5.3	5.8

* Referred to original inoculum as 100.

effected a net decrease in the number of viable organisms was 0.024 microgram per cc.; and essentially the maximal rate of killing was observed at 0.064 microgram per cc. At the maximally effective concentration it required from 2.5 to 5.1 hours to kill 99.9 per cent of the organisms, averaging 3.7 in the 5 experiments; and 0.004 to 0.072 per cent were still viable after 6 hours.

Of particular interest was the indication in some, but not all, of the experiments with this strain of pneumococcus of a slight zonal effect similar to that previously described for some strains of streptococci. This is evident in Table VIII.

Seven strains of pneumococci, of as many different types, were tested by the

same technic. As shown in Fig. 8, 6 of the 7 strains did not differ significantly with respect to either the effective concentrations of penicillin or the optimal rate of killing. One strain, a Type XI organism, was much less sensitive to penicillin, and was killed more slowly, than the other 6. The maximally effective concentration was 1 to 16 micrograms instead of 0.064; and the maxi-

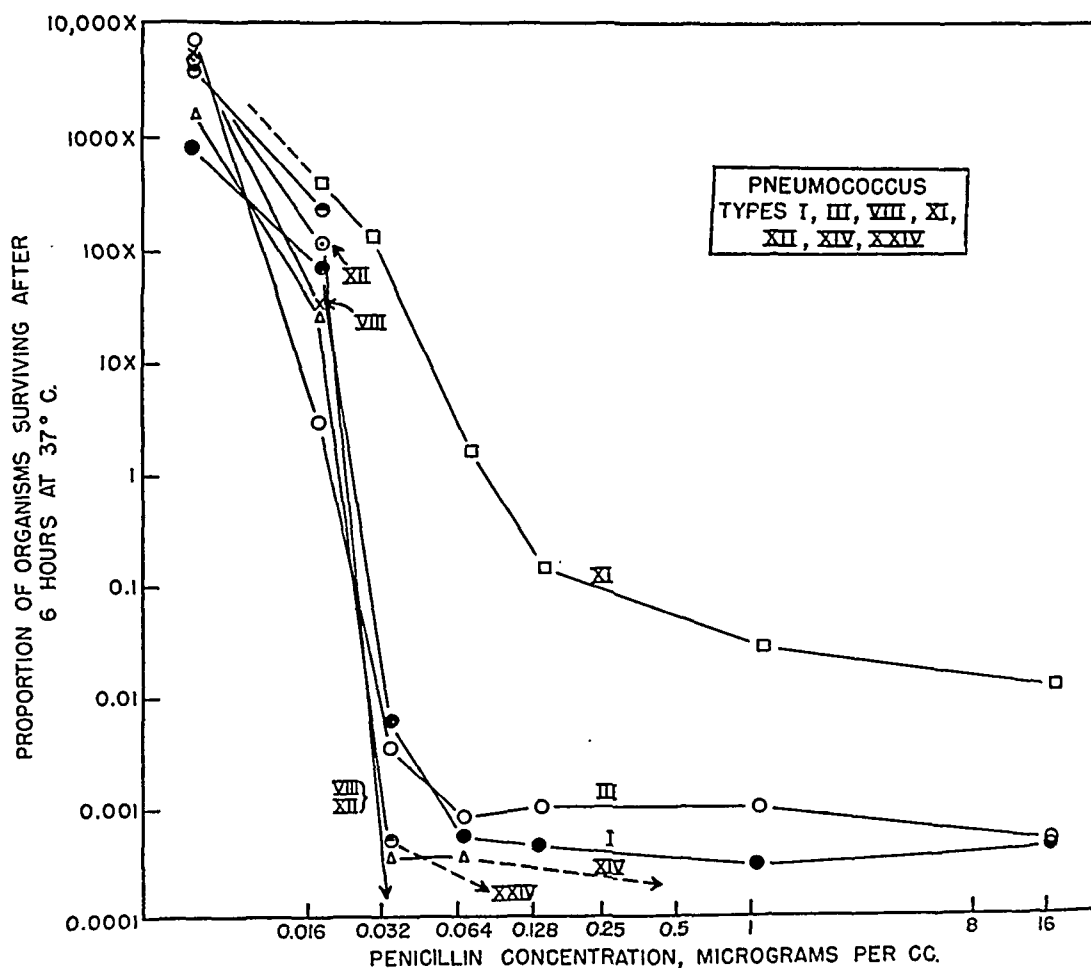


FIG. 8. The effect of the concentration of penicillin G on the proportion of organisms surviving after 6 hours at 37°C. (*Diplococcus pneumoniae*, types I, III, VIII, XI, XII, XIV, and XXIV).

mal rate at which the organisms could be killed by penicillin was also low, 1.3 per cent of the organisms surviving after 6 hours' exposure to 16 micrograms per cc.

D. Staphylococcus aureus and Staphylococcus albus

An experiment with a single strain of *Staphylococcus aureus* is summarized in the 2 sections of Fig. 9. As shown in Fig. 9 a, as the concentrations of

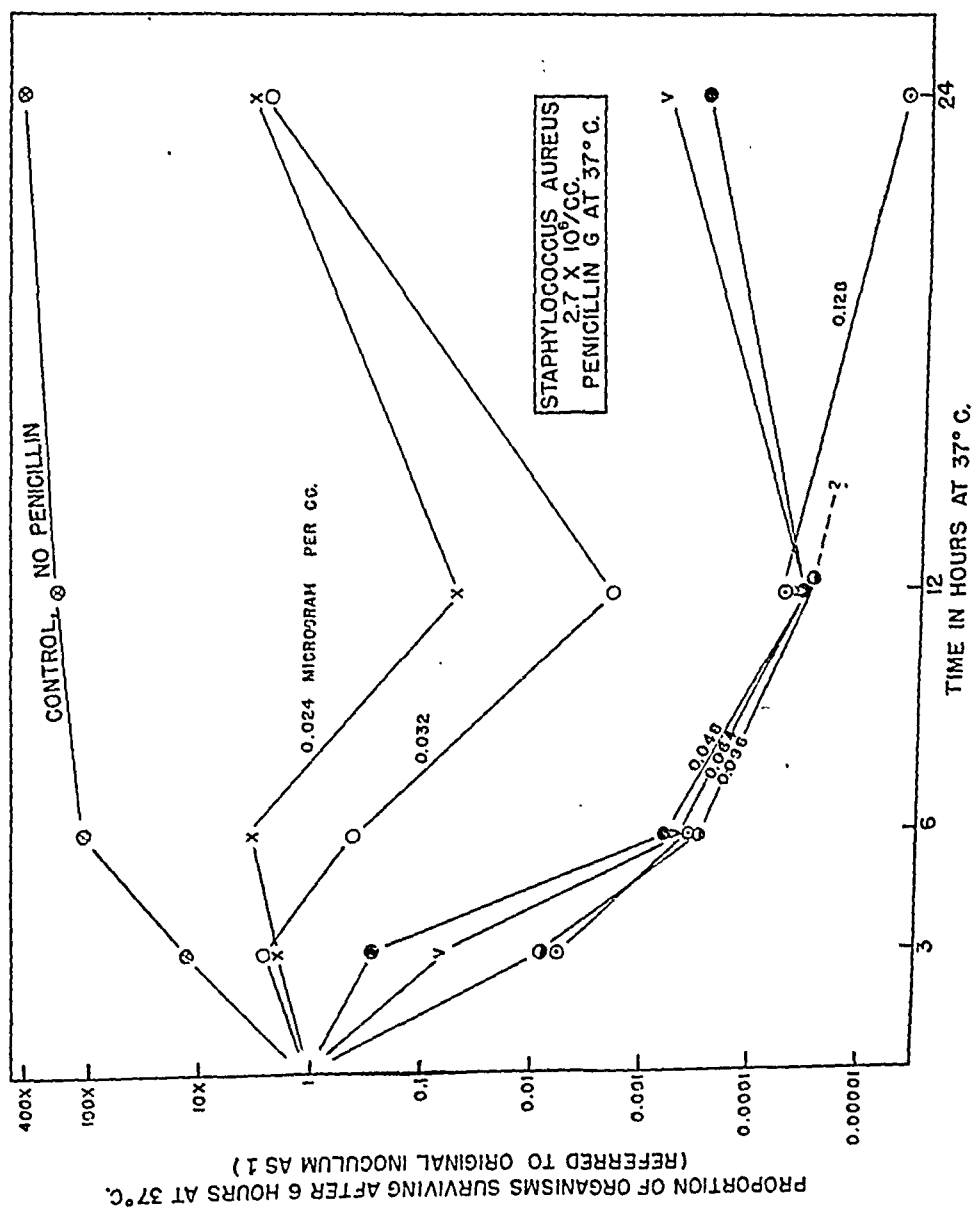
penicillin were increased up to the optimal concentration of 0.096 microgram per cc., there was a progressively more rapid bactericidal action. However, when the concentration of penicillin was increased beyond that optimal level, this trend was reversed, and the rate at which the organisms were killed fell off progressively (*cf.* Fig. 9 *b*). At concentrations of 1 microgram per cc., there were 110 times as many survivors after 6 hours as there were at 0.1 microgram, and it required 19 hours instead of 5 to kill 99.9 per cent of the organisms. Qualitatively similar results were obtained in every one of 5 experiments with this particular strain.

Of a total of 9 strains of staphylococci tested, 7 of *Staphylococcus aureus* and 2 of *Staphylococcus albus*, in 2 there was a well defined concentration of penicillin which was maximally effective, with diminished activity at concentrations in excess of that optimum; and in 2 others the zone phenomenon was less pronounced but none the less definite and reproducible (Table IX). With the other 5 strains the rate of bactericidal action increased with the concentration of penicillin to a maximum which was thereafter unaffected even by a 1000-fold increase.

It is to be noted in Fig. 9 *a* that at borderline concentrations of penicillin (0.024 and 0.032 microgram per cc. in that experiment) an initial decrease in the number of viable organisms was followed by an increase, the number of organisms ultimately reaching the same level attained in the control tube. This phenomenon was relatively uncommon with the group A streptococci, pneumococci, and treponemata, and then only within a narrow threshold range of penicillin concentrations; it was observed not infrequently with *Streptococcus fecalis*; but was a regular occurrence in experiments with staphylococci. In some of the experiments, as in that of Fig. 9 *a*, this remultiplication of organisms became manifest within a few hours, and at a time when only a relatively small proportion had been killed; in other experiments the remultiplication began only after 24 or 48 hours, sometimes after more than 99.99 per cent of the organisms had been killed. In the case of staphylococci, and unlike any of the other organisms here studied, the daughter cells of the last few resistant survivors were found to have a significantly increased resistance to the action of penicillin. It is therefore probable that remultiplication began when the average multiplication rate of the resistant survivors (which presumably developed as a mutation in the course of the experiment (7, 12)) exceeded the rate at which those surviving organisms could be killed by penicillin.

E. Treponema pallidum (Reiter)

One of 5 similar experiments with the cultured Reiter strain of so called *T. pallidum* is summarized in Table X, and a second in Fig. 10. In those experiments, a concentration of 0.016 microgram per cc. significantly decreased



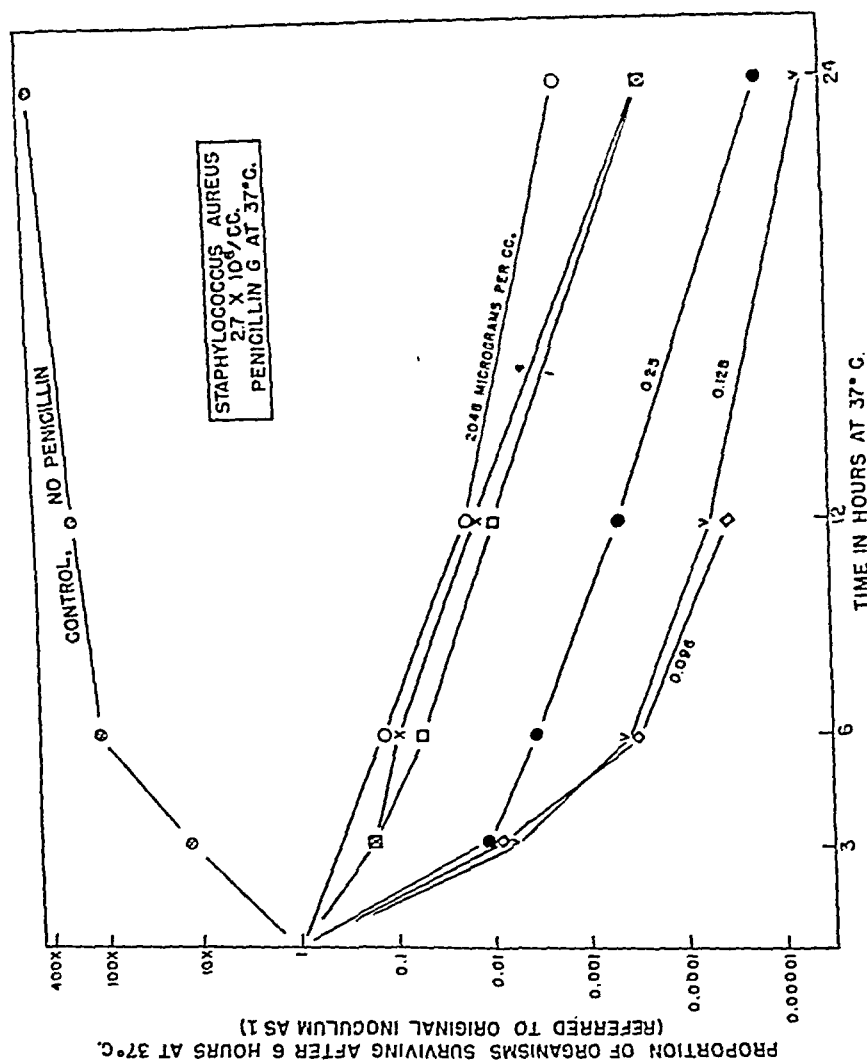


TABLE IX

The Susceptibility to Penicillin G of 9 Strains of Staphylococcus (Strains M14 and M15 Were Staphylococcus albus; the Other 7 Were Staphylococcus aureus, 3 of Which Showed a Paradoxical Zonal Susceptibility (Slower Rate of Killing at Higher Concentrations of Penicillin))

Strain No.	Concentration of penicillin (micrograms per cc.) which			Zone phenomenon at higher concentrations	Percentage* of organisms surviving after 6 hrs. exposure to		Time required to kill 99.9 per cent of organisms* at	
	reduces growth	effects net reduction in no. of viable organisms	kills organisms at maximal rate		optimal concentration of penicillin	256 micrograms per cc.	optimal concentration of penicillin	256 micrograms per cc.
							hrs.	hrs.
Smith	0.016-0.032	0.032	0.064-0.128	+	0.036	13	5	27
M10	0.016	0.032	0.064-0.14	+	0.08	3	5.8	13‡
M12	0.016	0.064	0.128	+	0.07	3.2	5.8	9.7
M15	0.016	0.032	0.128	+	0.23	8.0	9	23
F10	0.016	0.064	0.25	0	7	18	16	21
M11	0.25	1	1-16§	0	6	6‡	12	11
B	0.016	0.032-0.064	0.064	0	0.19	0.69	8	10
M13	0.032	0.128	0.25-1	0	1.1	0.54	11	11
M14	0.016	0.032	0.064-0.128	0	0.9	3.9	23	19

* Results in single illustrative experiment.

‡ 2,048 micrograms per cc.

§ An initial decrease was followed by late regrowth at concentrations of 1, 4, and 16 micrograms (*cf.* text) but not at higher concentrations.

TABLE X

The Rate at Which T. pallidum (Reiter) Is Killed at Varying Concentrations of Penicillin G

(Initial number of organisms in reacting mixture = 750,000 per cc.)

Time at 37°C.	Concentration of penicillin G, <i>micrograms per cc.</i>						Concentration of penicillin* (<i>micrograms per cc.</i>) which		
	0	0.016	0.064	0.25	1	256	reduced growth	effected net reduction in No. of viable organisms	killed organisms at maximal rate
	Percentage‡ of organisms still viable after indicated time period								
<i>hrs.</i>									
6	—	127	73	27	7	11	0.016	0.032-0.064	1-4
24	370	54	1.5	0.54	0.26	0.13			
48	1,450	—	0.28	0.038	0.017	0.015			
Time required to kill 99.9 per cent of organisms, <i>hrs.</i>	∞	?	65±	39	32	27			

* Based on this and 4 similar experiments.

‡ Referred to original inoculum as 100.

the net rate of multiplication; the minimal concentration with a net bactericidal action was 0.032 microgram per cc.; and almost the maximum effect was produced by a concentration of 0.25 to 1 microgram per cc. Thereafter, a 2000-

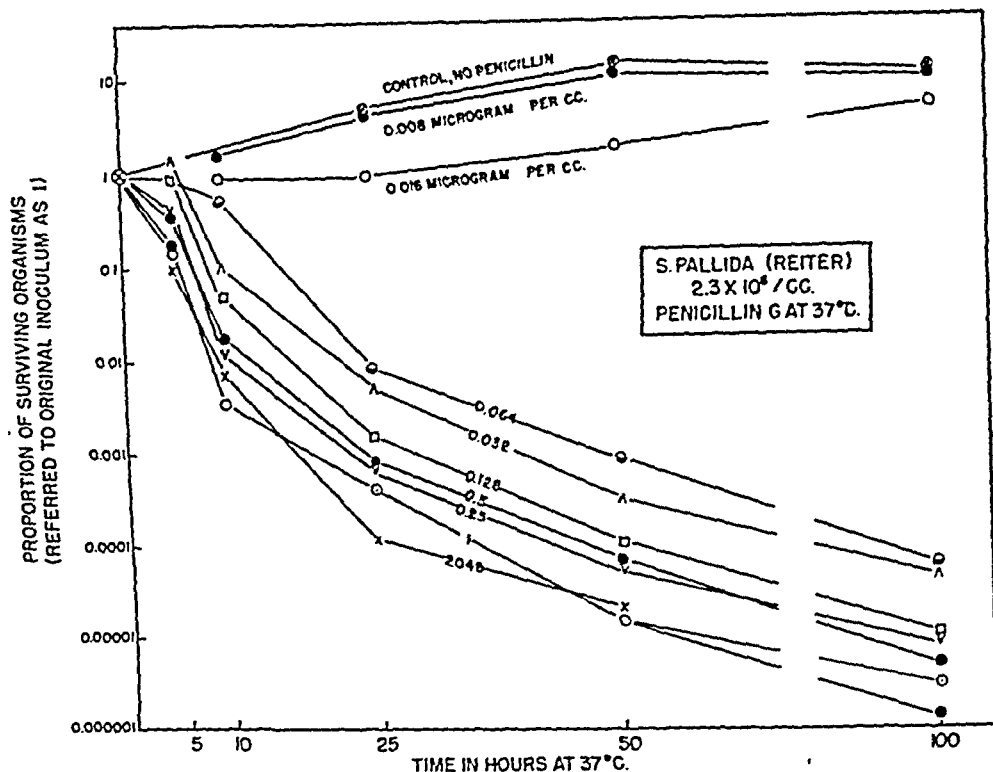


FIG. 10. Effect of the concentration of penicillin G on the rate at which treponemata (Reiter strain) are rendered non-viable *in vitro*.

fold increase in penicillin concentration, up to 2048 micrograms per cc. only slightly accelerated the rate at which the organisms were killed. These results with crystalline penicillin G agree qualitatively and quantitatively with those obtained in previously reported studies (2) with a sample of commercial penicillin known to contain significant amounts of the other penicillin species.³

³ With amorphous penicillin, longer periods of time were necessary to effect 99.9 per cent reduction than with crystalline G; and the maximally effective concentration was significantly higher. Further, with commercial penicillin there was a greater tendency for the organism to grow out after an initial decrease. These differences may reflect the greater stability of crystalline penicillin in comparison with that of the amorphous commercial product, under the conditions of the experiment.

The slow rate at which these treponemal cultures were killed, as compared with for example *Streptococcus pyogenes*, is particularly to be noted (compare Fig. 10 with Fig. 1, or Table X with Table I). To kill 99.9 per cent of the treponemata at the maximally effective level of 1 microgram per cc. required from 23 to 33 hours, against 1.4 to 2.2 hours for the C-203 strain of *Streptococcus pyogenes*; and the proportion of organisms surviving after 6 hours was 1 to 10 per cent, as compared to 0.002 to 0.01 per cent for the streptococcus.

III. THE DIFFERENCES IN RESISTANCE TO PENICILLIN AMONG BACTERIAL POPULATIONS

The susceptibility of bacteria to penicillin may be defined in terms of two variables: the concentrations of the drug which are necessary to kill the bacteria, and the rate at which the organisms are killed at these concentrations. Although the former factor has been stressed in most previous studies, the maximal rate at which a given bacterial strain can be killed by penicillin may also be of major therapeutic significance. Both factors varied widely among the strains here studied, and there was no necessary correlation between the two.

A. Differences in the "Effective" Concentrations of Penicillin for Different Bacterial Species and Strains.—Three "effective" concentrations of penicillin have been here defined for a number of bacterial species and strains (*cf.* section *a* of Table XI). (*a*) The first and lowest is that concentration of drug which suffices only to reduce the rate at which the net number of viable organisms increases. (This concentration may be of therapeutic significance if it should be proven that it renders bacteria *in vivo* more vulnerable to the body's natural humoral or cellular defense mechanisms, even though it does permit multiplication at a diminished rate *in vitro*.) (*b*) At a somewhat higher concentration, penicillin causes a slow progressive reduction in the number of viable organisms. This approximately equals the concentration which prevents visible growth *in vitro*, and thus approximates the "sensitivity" of a given strain to penicillin as ordinarily determined (1-4, 8, 13, 14). It is not, however, the concentration at which the organisms are killed at the maximal rate *in vitro*. (*c*) The latter maximally effective concentration was usually 2 to 20 times the minimal concentration which was effectively bactericidal, and probably represents the concentration which should be maintained at the focus of infection in order to kill the largest number of organisms in the shortest possible time. Even a 32,000-fold increase beyond this maximally effective concentration did not further increase the rate of bactericidal action of penicillin. Unless high concentrations of penicillin can be shown to have an effect *in vivo* over and above their direct bactericidal action, then in the case of most of these organisms a penicillin level of for example 0.1 to 1 microgram per cc. maintained at the focus of infection would be expected to be just as effective therapeutically as concentrations of 10, 100, or 1000 micrograms per cc. Extremely large doses of penicillin

TABLE XI

*The Susceptibility of a Number of Organisms to Penicillin**(a) The Effective Concentration of the Drug**(b) The Presence of a Zone Phenomenon (Paradoxically Retarded Bactericidal Action) at High Concentrations of Penicillin**(c) The Time Required to Kill 99.9 Per Cent of the Organisms at Optimal and Excessive Concentrations**(d) The Proportion of Organisms Surviving after Exposure for 6 Hours to Optimal and Excessive Concentrations of Penicillin*

Species		No. of strains studied	(a) Concentrations of penicillin G (micrograms per cc.) which			(b) No. strains showing zone phenomenon (slower bactericidal action at higher concentrations of penicillin)		(c) Time required to kill 99.9 per cent of organisms at		(d) Percentage of organisms surviving after 6 hrs.* exposure to	
			decreased net rate of multiplication	slowly killed organisms	killed organisms at maximal rate	Yes	No	optimum concentrations of penicillin	256 micrograms per cc. and higher	optimal concentration of penicillin	256 micrograms per cc. and higher
β -hemolytic streptococci	Lancefield Group A	5	0.004-0.008	0.016	0.032-0.064	0	5	hrs. 2-8	hrs. 2-5	0.002-0.6	0.004-0.25
	Group B	4	0.016	0.032-0.064	0.064-0.128	4	0	4-10	15-23	0.03-0.56	1.1-44
	Group C	4	0.004	0.016	0.032	3	1	4-9	13, >9, 21, 14	0.011-0.27	0.19-15
	M31*	1	1	4	4-8	1	1	4 11-17	5 54	0.02-0.08 0.17-0.6	0.02-0.05 19-80
α -hemolytic streptococci	<i>Fecalis</i>	7	1	3-4	4-6	5	2	4.6-5.2 48	30-40 48	0.029-0.062 13, 48	23-44 7, 50
	Others	5	0.016-0.032	0.032-0.064	0.128	3	2	6.5-11 2.4, 25	26-45 2.4, 29	0.12-0.67 0.03, 16	11-44 0.05, 20
Staphylococci	<i>S. aureus</i>	9	(8) 0.016-0.032	0.032-0.064	0.064-0.128	4	5	5-9	13-27	0.07-0.9	3-22
	and <i>S. albus</i>		(1) 0.25	1	16	5	5	8-17	10-21	1.1-8.5	0.54-18
Pneumococci	Types 1, 3, 8, 12, 14, 24	6	0.006-0.016	0.032	0.032-0.064	1†	5	2.5-4.4‡	4.6-6‡	0.004-0.058	0.01-0.075
<i>T. pallidum</i> (non-pathogenic Reiter strain)		1	0.016	0.032-0.064	1-4	0	1	23-42	17-27	6.4-17	11-15

* Although this strain reacted with a group C typing serum, it was a lanceolate organism which grew in pairs or occasional short chains, liquefied gelatin, and fermented lactose, salicine, mannite, trehalose, and sorbitol. It probably falls in the *Streptococcus zymogenes* group.

† 512 micrograms per cc. instead of 256.

‡ Values for Type I strain only.

would be of therapeutic advantage only to the degree that they provided this maximally effective concentration for longer periods of time, and not by virtue of the higher absolute levels attained.

The three effective levels of penicillin for the various bacterial species and strains here studied are summarized in section *a* of Table XI. In Figs. 11 and 12, the lowest point in each curve indicates the maximally effective concentration of penicillin for a single strain of each species, *i.e.* the concentration at

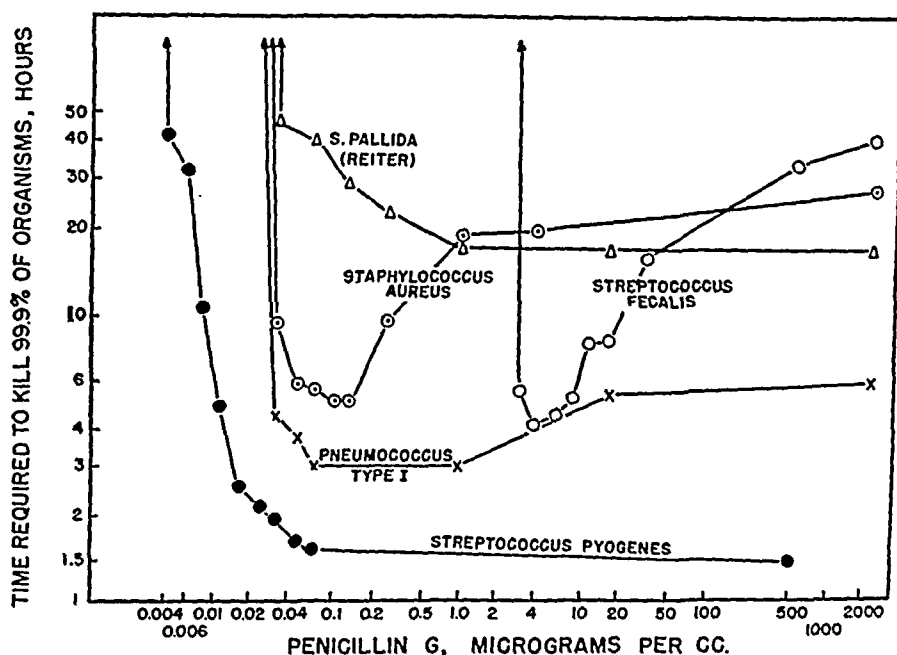


FIG. 11. Effect of the concentration of penicillin on the time required to kill 99 per cent of the organisms. Illustrative single experiment with single strain each of *T. pallidum* (Reiter), *Staphylococcus aureus*, *Streptococcus fecalis*, *Diplococcus pneumoniae* Type I, and *Streptococcus pyogenes*.

which for example 99.9 per cent of the organisms were killed in the shortest time (Fig. 11), or at which there was the smallest proportion of viable survivors after 6 hours for example (Fig. 12).

The large differences in the maximally effective concentrations for different species of bacteria are well known. Within a single species, different strains usually had approximately the same sensitivity. There were, however, occasional striking disparities (*cf.* the relatively resistant staphylococcal strain M11 in Table IX, and the Type XI pneumococcus in Fig. 8; the identification of the discrepant M31 strain in Table V as a group C streptococcus was questionable).

The Varying Resistance to Penicillin of Individual Organisms in the Same Bacterial Culture: Within the same bacterial culture, individual organisms

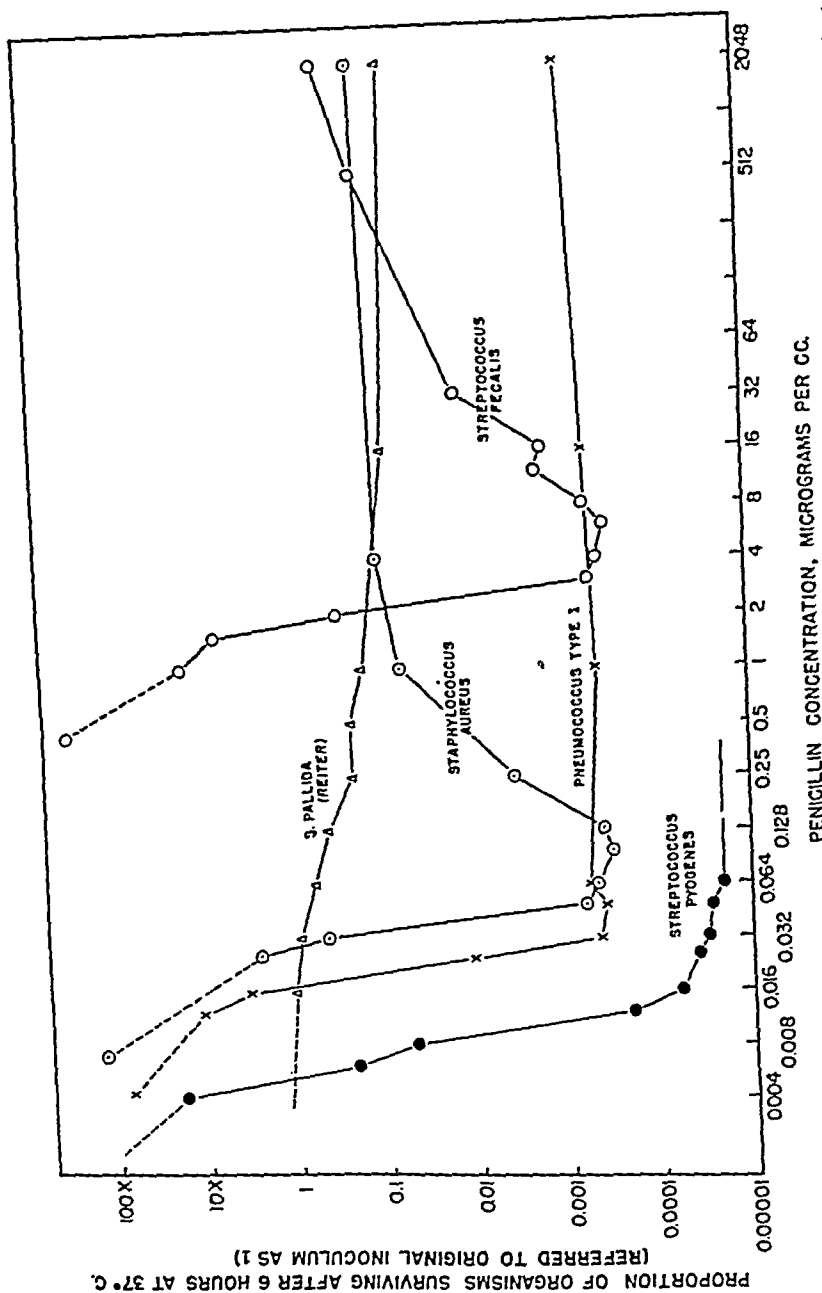


Fig. 12. Effect of the concentration of penicillin on the proportion of organisms surviving after 6 hours at 37°C. Illustrative single experiment with single strain each of *T. pallidum* (Reiter), *Staphylococcus aureus*, *Streptococcus fecalis*, *Diplococcus pneumoniae* Type I, and *Streptococcus pyogenes*.

also varied in their susceptibility to penicillin. At threshold concentrations of the drug only a fraction of the organisms might be killed; and an initial decrease in the number of viable organisms which reflected the death of the more susceptible, was often followed by the rapid multiplication of the relatively more resistant organisms.⁴ All the organisms would, however, be killed by higher concentrations of penicillin. A 4-fold difference in concentration constituted the usual range of variation between the sensitivity threshold of the most susceptible and the most resistant organisms in a given suspension. The factor underlying this difference remains to be determined (*cf.* Foster (10); Foster and Wilker (11)).

B. The Widely Varying Maximal Rates at Which Different Strains of Bacteria or Different Organisms within the Same Strain Can Be Killed by Penicillin.—The concentration of penicillin which suffices to kill a given bacterial strain *in vitro* affects what may be called the *intensity* of treatment; *i.e.*, the dosage of penicillin to be administered, and the frequency with which that dose should be repeated. The pronounced differences between bacterial species in this respect have been stressed by numerous workers. It is, however, apparent from Table XI and Figs. 11 and 12 that there are also pronounced differences in the maximal rate at which the various bacterial species can be killed by penicillin. This is indicated by the varying vertical position of the minima on the curves of Figs. 11 and 12. Thus, after 6 hours' exposure to the most effective concentration of penicillin, 1 to 10 per cent survived in the case of the Reiter treponeme (4 experiments), but <0.0004 per cent in the case of the F24 strain of group A β -hemolytic streptococci, a difference in this respect of more than 2500-fold. Similarly, the times required to kill 99.9 per cent of the organisms in suspensions of these 2 strains were respectively 17 to 37 and 2 to 3 hours. Even within the same species, different strains varied markedly. With the M4 strain of *Streptococcus fecalis*, only 0.029 per cent survived a 6 hour exposure to the optimally effective concentration of penicillin, but 48 per cent of strain F1 survived under similar conditions; and the times required to kill 99.9 per cent were 4.6 and >48 hours, respectively. These differences in the susceptibility of the bacterial strains to penicillin in terms of the rate at which they can be killed may well affect the length of time for which treatment must be continued to effect cure. An organism of which 99.9 per cent can be killed in for example 3 hours will probably be more susceptible to treatment than one which requires 48 hours' exposure even at maximally effective concentrations of the drug.

There was no *necessary* correlation between the concentration of penicillin necessary to kill a given organism, and the maximum rate at which that or-

⁴ This may be observed in the absence of demonstrable mutation or adaptation. The daughter cells of these relatively resistant survivors may have the same distribution of resistance as the original culture.

ganism could be killed by the drug. This is indicated by the wide scatter of the points in Fig. 13. Organisms exquisitely sensitive to penicillin in terms of the low concentrations which sufficed to kill the organisms at a maximal rate might be killed only slowly at that optimum concentration (cf. Δ and \blacktriangle in lower left of Fig. 13); while others killed only by high concentrations might be killed quite rapidly (cf. \times in upper right portion of figure). Nevertheless, Fig.

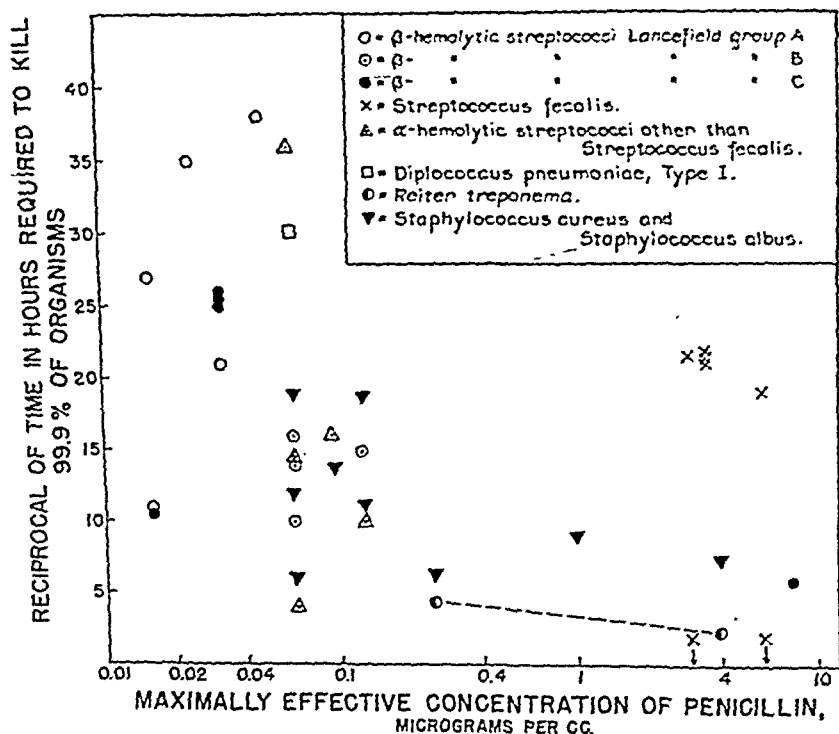


FIG. 13. The correlation between the maximally effective concentrations of penicillin for various bacterial species and the rate at which the organisms are killed at those maximally effective levels.

13 does suggest a rough correlation between the concentrations of penicillin maximally effective against the various strains, and the rates at which the organisms could be killed by those maximally effective concentrations. By and large, the more resistant a strain in terms of the amounts of penicillin required, the more resistant it was also in terms of the rate at which the organisms were killed, and in the times required to sterilize a given suspension.

C.—Yet a third factor which affects the "curability" of an infection with penicillin, over and above the effective concentrations of the drug, and the rate at which the particular strain of organism can be killed, is the initial number

of bacteria in the infected host. There are several reports which describe the bactericidal action of penicillin as a function of the number of organisms (15-17). In our own experiments, however, the initial number per cubic centimeter usually had no material effect on the rate of bactericidal action of penicillin. Although there were occasional exceptions (*cf.* staphylococcus experiment in Fig. 14) essentially the same proportion was usually killed within a given time period whether the initial number was; *e.g.*, 1000 or 1,000,000 per cc. (*cf.* Fig. 14).

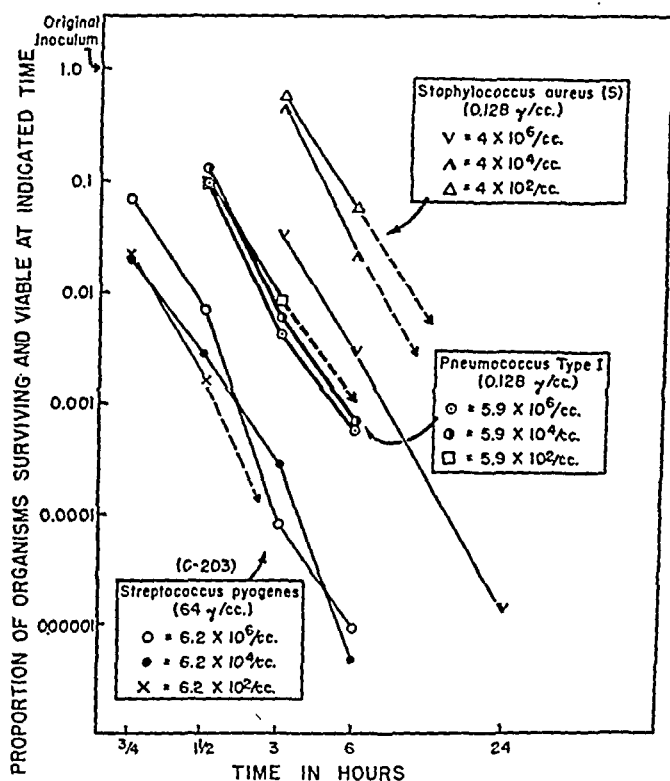


FIG. 14. The effect of the initial number of organisms on the rate at which bacteria are killed by maximally effective concentrations of penicillin.

It is nevertheless clear that the more organisms which are initially present in a culture or in an infected host, the longer will be the times required to effect sterilization or cure. Of an initial 1000 organisms, only > 99.9 per cent need be killed;⁵ but with 1,000,000 organisms that proportion becomes > 99.9999 per cent. A longer time is then required, not only because a larger proportion must be disposed of, but also because the last few highly resistant organisms require a disproportionately long exposure to the drug (*cf.* Figs. 1, 9, 10, and 14). It usually took as long or longer to kill the last 0.01 per cent of the organisms as

⁵ Assuming that the persistence of 1 organism implies the persistence of infection: the argument is not affected whatever one sets as the arbitrary threshold of cure.

it did to kill the entire first 99.99 per cent; and there was usually an 8-fold difference in the times required to sterilize suspensions of 1000 and 1,000,000 organisms.

It is perhaps unnecessary to point out that the foregoing considerations are based on *in vitro* experiments, and that the participation of the host may alter these relationships not only quantitatively, but perhaps also qualitatively. However, such data as are available indicate that these inferences may be valid also *in vivo*. In syphilitic rabbits inoculated with 20, 2000, and 200,000 organisms, and treated 4 days later with a single injection of penicillin suspended in oil and beeswax, the doses necessary to abort infection in half the animals have been shown to be approximately 200, 500, and 3500 units per kilo, respectively (18). The larger the inoculum, the longer was the time period for which the effective concentrations of penicillin had to be maintained in order to kill the very last organism and thus abort the infection; and the correspondingly larger was the dose of penicillin which had to be administered. Even more striking correlations between the number of organisms in the infected animal, and the curative dose of penicillin (*i.e.*, the period of time for which effectively bactericidal levels must be maintained) have more recently been observed in experimental pneumococcal infection in mice treated with penicillin in aqueous solutions (19, 20).

The foregoing discussion involves the several factors of effective penicillin concentration, the varying rate at which different bacterial strains can be killed even at maximally effective levels of the drug, the number of organisms to be killed, and the total time for which the bacteria are exposed to effective concentrations. A corollary to these factors is the therapeutic problem as to whether the exposure to penicillin should be continuous, and the penicillin concentrations *in vivo* therefore sustained at effectively bactericidal levels, or whether the penicillin levels may be intermittently allowed to fall below those effective levels without prejudicing the outcome of the treatment. Experiments on this point, with particular reference to the time required for various bacteria to recover from the toxic effects of penicillin *in vitro* and *in vivo*, will be described in a following paper ((19); *cf.* also (21)).

IV. ZONE PHENOMENON: THE PARADOXICALLY REDUCED ACTIVITY OF HIGH CONCENTRATIONS OF PENICILLIN AGAINST CERTAIN BACTERIA

A puzzling aspect of the present experiments was the demonstration that for many, but not all, strains of both α - and β -hemolytic streptococci, and for many strains of staphylococci, there was an optimal concentration of penicillin in excess of which the organisms were killed less rapidly than they were at the lower concentration (*cf.* (9)). This is evident in the curves of Figs. 4, 5, 6, 7, 11, and 12, in which the well defined minima represent the optimal concentrations of penicillin. With a Type I strain of pneumococcus this zone was observed only irregularly, and was not pronounced. With the other bacterial

species and strains here studied, there was no zonal effect; instead, the rate of bactericidal action reached a maximum at a concentration of penicillin characteristic of the particular strain, and which was not further affected even by a 2000- to 32,000-fold increase in penicillin concentration.

With the organisms which showed this zonal susceptibility, the maximally effective range was sometimes extraordinarily narrow. Thus, with the S strain of *Streptococcus fecalis* (page 110), 2 micrograms per cc. did not suffice to sterilize the culture, and 4 to 6 micrograms were maximally effective. When the penicillin concentration was increased to as little as 8 micrograms per cc., it was significantly less effective, and it became progressively less so the higher the concentration. Similarly, in the case of the Smith strain of *Staphylococcus aureus* (page 114), 0.032 microgram per cc. usually failed to sterilize the culture, 0.064 to 0.096 microgram per cc. were maximally effective, and an increase to as little as 0.128 to 0.25 microgram per cc. significantly retarded the rate of death of the organisms.

The zone phenomenon here described has not yet been satisfactorily explained. (a) It was not necessarily related to the production of free penicillinase. Although the M11 zonal strain of *Staphylococcus aureus* did inactivate penicillin in culture, the S strain of *Staphylococcus aureus*, the 5 strains of *Streptococcus fecalis*, and the M31 strain of β -hemolytic streptococci, all of which showed a definitely retarded bactericidal action at high concentrations of penicillin, had no effect on the drug when they were permitted to grow in solutions containing less than the inhibitory concentration (0.25 to 0.5 microgram per cc.) (cf. Table XII).

(b) It seems unlikely that the zones were due to the presence in penicillin of a trace of impurity which inhibited the action of the drug. Garrod (23) had noted the retarded action of penicillin at high concentrations against staphylococci, but ascribed this to the presence of impurities, and stated that this zonal effect was not obtained with penicillin of about 85 per cent purity (cf. also reference 24). However, the data here reported with crystalline penicillin G, some lots of which were, on the basis of counter-current distribution diagrams, reported to be more than 95 per cent pure, indicate that this zonal effect is actually a property of the drug and not due to associated impurities. It is further difficult on the latter basis to explain the fact that the inhibitory effect became evident at a level which differed so markedly among different bacterial strains, and corresponded so regularly to the range of effective concentrations. Further, different lots of crystalline penicillin G, from different manufacturers, behaved identically with respect to the zone phenomenon, and it was observed with penicillins K and X, as well as G.

A clue to the nature of this paradoxical zone may be provided by the observation that the degree to which the bactericidal action of penicillin was retarded at high concentrations varied not only with the concentration of the drug, but also with the number of bacteria. This point is under present study.

TABLE XII

Showing that the Zonal Susceptibility to Penicillin Is Not Necessarily Related to the Ability of the Particular Bacterial Strain to Destroy Penicillin

(Of 8 zone-sensitive organisms tested, only 1 was found to produce extracellular penicillinase)

Species	Strain No.	Minimal bactericidal concentration of penicillin, micrograms per cc.	Concentration of penicillin added to medium, micrograms per cc.	Inoculum, No. per cc. $\times 10^6$	Degree of bacterial multiplication after			Residual penicillin activity after			Conclusion
					6 hrs. at 37°C.	24 hrs. at 37°C.	48 hrs. at 37°C.	6 hrs. at 37°C.	24 hrs. at 37°C.	48 hrs. at 37°C.	
Group A hemolytic streptococcus	M31	4	0.25	6.0	44X			per cent 100			No significant inactivation of penicillin by growing organisms
			0.25	1.2	277X	736X		75	75		
<i>Staphylococcus aureus</i>	S	0.032-0.064	0.025	2.1	1,575X		2,560X	100		63	" "
	M11	4-16	0.25 0.1	1.1 0.8	14X 95X			< 5 < 13			Essentially complete inactivation of penicillin in 6 hrs.
<i>Streptococcus fecalis</i>	H		0.5	4.7 3.4 1.3	40X 84X 233X	82X 148X 295X	76X 188X —	100 100 75	83 100 100	83 50	No inactivation of penicillin by growing organisms
	M1		0.5	1.2	178X	342X		90	75		" "
	M3	3-4	0.5	0.6	7X	142X		71	75		" "
	M5		0.5	1.3	103X	156X		100	75		" "
	M6		0.5	1.3	153X	280X		100	100		" "
Controls in sterile broth			2					100			
			0.5					82	60		
			0.4					83	67		
			0.25					100	100	63	

In the treatment of infections caused by these zone-sensitive organisms, the initial blood and tissue levels provided by large doses of penicillin may be far in excess of those which are optimally effective *in vitro*. Those high concentrations may be correspondingly less effective *in vivo*. Although the blood and tissue levels eventually fall to the optimal concentration, they remain in that

optimally effective range for a relatively short period of time, because of the rapid rate at which penicillin is excreted. In the treatment of infections caused by such zone-sensitive bacteria, a number of relatively small doses of penicillin repeated at appropriate intervals (22), or the slowly absorbed suspensions of penicillin in oil and beeswax, may therefore be more effective therapeutically than extremely large injections which maintain the tissue concentrations at excessively high levels. The ideal method of treatment in such infections may well be a continuous infusion at a rate designed to maintain, at the focus of infection, a concentration of penicillin approximating that which is maximally effective for the particular organism.

SUMMARY

1. The concentrations of penicillin G which (a) reduced the net rate of multiplication, (b) exerted a net bactericidal effect, and (c) killed the organisms at a maximal rate, have been defined for a total of 41 strains of α - and β -hemolytic streptococci, *Staphylococcus aureus* and *Staphylococcus albus*, *Diplococcus pneumoniae*, and the Reiter treponoma.

2. The concentration which killed the organisms at a maximal rate was 2 to 20 times the minimal effective level ("sensitivity" as ordinarily defined). With some organisms, even a 32,000-fold increase beyond this maximally effective level did not further increase the rate of its bactericidal effect. However, with approximately half the strains here studied (all 4 strains of group B β -hemolytic streptococci, 4 of 5 group C strains, 5 of 7 strains of *Streptococcus fecalis*, 2 of 4 other α -hemolytic streptococci, and 4 of 9 strains of staphylococci), when the concentration of penicillin was increased beyond that optimal level, the rate at which the organisms died was paradoxically reduced rather than increased, so that the maximal effect was obtained only within a relatively narrow optimal zone.

3. There were marked differences between bacterial species, and occasionally between different strains of the same species, not only with respect to the effective concentrations of penicillin, but also with respect to the maximal rate at which they could be killed by the drug in any concentration. Although there was a rough correlation between these two factors, there were many exceptions; individual strains affected only by high concentrations of penicillin might nevertheless be killed rapidly, while strains sensitive to minute concentrations might be killed only slowly.

4. Within the same bacterial suspension, individual organisms varied only to a minor degree with respect to the effective concentrations of penicillin. They varied strikingly, however, in their resistance to penicillin as measured by the times required to kill varying proportions of the cells.

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THE REACTIVITY OF VARIOUS HUMAN SERA WITH MUMPS COMPLEMENT FIXATION ANTIGENS*

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A complement fixation test for mumps was first described by Enders and his coworkers (1, 2). This test, which employed suspensions of parotid glands from monkeys infected with mumps virus, was found very useful since antibodies could be detected in all patients following an attack of the disease. It proved particularly valuable as an aid in the diagnosis of some of the more unusual manifestations of infection with the virus of mumps, such as meningoencephalitis in the absence of preceding or concurrent parotitis (3). Extensive studies on the correlation between the results of the complement fixation test and resistance to mumps (4) showed that the disease occurred practically always in individuals whose sera gave negative reactions prior to infection. A positive test was obtained in 72 per cent of the subjects admitting parotitis at some time in the past. It also was found positive in 42 per cent of individuals who failed to reveal previous attacks of mumps. This finding, indicating the frequency of inapparent infections with mumps virus, was substantiated by the demonstration in some exposed individuals of the formation of complement-fixing antibodies in the absence of clinical signs of disease.

The adaptation of mumps virus to the chick embryo led to a more readily available source of antigen (5, 6). An analysis of various preparations of tissues and fluids derived from chick embryos infected with this agent revealed that they contained at least two serologically distinct complement fixation antigens (7). One of these was found to be closely linked with the virus and present predominantly in the amniotic and allantoic fluids; the other was smaller in size and demonstrable mainly in the infected tissues; *i. e.*, in the amniotic and allantoic membranes. The virus-bound or "V" antigen could be differentiated from the smaller soluble or "S" antigen by serum cross-absorption technique, in that antibodies against V could be removed from human convalescent sera by absorption with this antigen, leaving most of anti-S in solution and, conversely, absorption with S left antibodies against V in the serum. This observation of antigenic differences was confirmed by the fact that human sera reacted to a varying extent with S and V preparations. The antigen

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derived from the infected monkey parotid gland, which had been used by the earlier workers, has not been compared as yet with the chick embryo materials. It is impossible, therefore, to state definitely at present whether it contained measurable quantities of both the V and S antigens or only one of them. Certain of the observations to be described point to dominance of the S antigen in suspensions of monkey parotid glands.

It is the purpose of this paper to summarize the results of an extensive analysis of the serological reactivity of human sera taken at various stages of infection. The results show that antibodies against S antigen appear earlier, as a rule, than those against V, and that anti-V remains measurable usually for a longer period than anti-S. Thus, it will be shown that both antigens have their place in the early diagnosis of mumps, whereas in the determination of susceptibility use of the V antigen only appears sufficient.

Materials and Methods

Preparation of Complement-Fixing Antigens.—The egg-adapted strain of mumps virus used for the preparation of antigens was obtained from Dr. John F. Enders in the 5th amniotic passage (6). It has been adapted to the allantoic sac of the chick embryo yielding allantoic fluids of high infectivity (10^8 ID₅₀/ml.). The antigens were prepared from allantoic fluids and chorioallantoic sacs of the 14th to 16th allantoic passages. Suitable numbers of 8-day-old chick embryos were inoculated with 0.5 ml. of infected allantoic fluid diluted in broth to 10^{-2} to 10^{-4} . The technic of inoculation has been described previously (7). After 5 days of further incubation of the eggs at 36–37°C. the allantoic fluids as well as the allantoic sacs were harvested aseptically. The fluids containing the virus-bound antigen (V) were dialyzed in sterile cellophane bags against 20 volumes of M/100 phosphate-buffered saline of pH 7.0 in order to remove most of the urates prior to irradiation with ultraviolet light by a technic previously described (8).

The allantoic sacs were thoroughly washed in sterile buffered saline solution, drained on sterile filter paper, and weighed. A 20 per cent suspension in buffered saline solution was made by emulsifying the tissue in a Waring blender for 3 minutes. After preliminary centrifugation of the suspension at 2,000 R.P.M. for 10 minutes, the supernatant fluid was subjected to high speed centrifugation at 20,000 R.P.M. for 20 minutes. The supernatant fluid obtained after this centrifugation served as soluble (S) antigen.

Control antigens were prepared according to the methods described above from uninfected chick embryos of the same age and usually from the same batch of eggs supplying the mumps preparations. The normal allantoic fluid gave positive complement fixation tests with human sera only very rarely. In these cases, the control antigens prepared from normal allantoic membranes, likewise, gave positive results. The normal allantoic fluid was omitted, therefore, in later tests.

The dialyzed infected allantoic fluid was irradiated in order to inactivate the virus, and the membrane antigen was treated in the same way. To all antigens 1:10,000 merthiolate was added as a preservative. They proved to be stable at 4°C. for at least 3 months.

Human Sera.—Blood was collected from patients¹ at varying stages of infection with mumps virus and permitted to clot. The serum was separated and inactivated at 60°C. for 20 minutes. In case reactions with the normal control antigen were encountered, a second

¹ The sera of 63 cases were studied in collaboration with Dr. Vera Oldfeld of the Epidemiskjukhuset, Stockholm, Sweden.

heating of the sera to 60°C. for 20 minutes frequently decreased this reactivity (2) but rarely removed it completely. The sera were stored in the frozen state at -10°C. until used for the tests.

Complement Fixation Test.—Twofold dilutions of the sera were prepared beginning with undiluted serum or 1:2 in cases where low or negative reactions were expected, and with correspondingly higher dilutions in convalescent specimens. Of each dilution 0.1 ml. was transferred to 4 tubes each. Thus, 4 series of increasing dilutions were obtained, the first set to receive saline solution (serum control); the second, mumps allantoic fluid (V antigen); the third, the supernatant fluid of normal allantoic sac (N antigen); and the fourth, the supernatant fluid of mumps allantoic sac (S antigen). The V and S antigens were used in optimal dilution; *i.e.*, the dilution giving the highest titer with a standard serum according to preliminary titrations. Such a standardization test is shown in Table I. The N antigen was diluted to the same extent as the S preparation, both showing approximately the same amount of nitrogen and phosphorus. Each antigen dilution was mixed with an equal volume of

TABLE I
Optimal Titration of S and V Antigens and Convalescent Serum

Serum 1:2	S antigen											V antigen						Sa- line
	Und.	1:2	1:3	1:4	1:6	1:8	1:12	1:16	1:24	1:32	1:48	Und.	1:2	1:3	1:4	1:6	1:8	
1:32	0	0	0	0	0	0	0	0	0	0	wk	0	0	0	0	(0)	tr	c
1:64	0	0	0	0	0	0	0	0	0	0	st	0	0	0	0	(0)	tr	
1:128	0	0	0	0	0	0	0	0	(0)	wk	ac	0	0	0	0	(0)	wk	
1:256	ac	wk	(0)	(0)	0	0	(0)	tr	wk	st	c	(0)	0	0	(0)	wk	ac	
1:512	c	c	c	(c)	(c)	(c)	(c)	c	c	c	c	tr	0	0	tr	st	(c)	
1:1024	c	c	c	c	c	c	c	c	c	c	c	ac	ac	ac	c	c	c	
Saline	c											c						

0 = no hemolysis; tr = trace; wk = weak; st = strong; ac = almost complete; c = complete hemolysis.

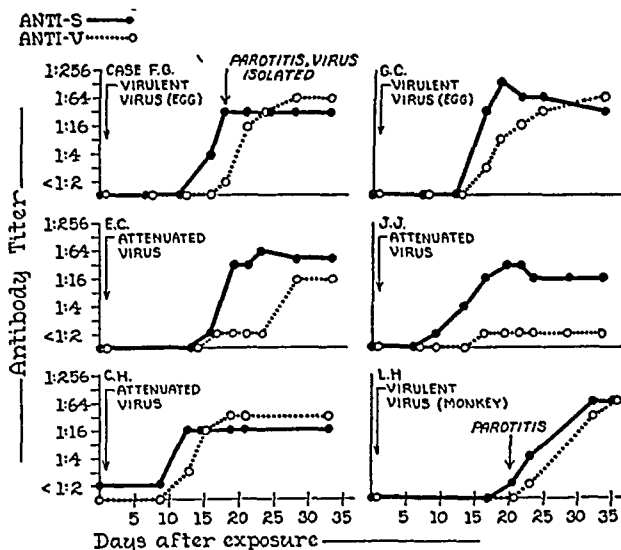
suitably diluted guinea pig complement and the mixture was then added to the corresponding tubes, in 0.2 ml. amounts. In large tests the antigen-complement mixtures were added by means of an automatic pipette. Sharp and Dohme "Lyovac" complement was used throughout.² It was adjusted to contain 1.5 minimal hemolytic units per 0.1 ml. After the primary incubation of the test at 37°C. for 1 hour, 0.2 ml. of sensitized sheep cells (2.5 per cent) was added by automatic pipette. The test was further incubated at 37°C. for 1 hour when readings were recorded. The last dilution of serum giving complete fixation of complement (no hemolysis) was considered to be the end point. All antibody titers are recorded as the initial dilution of serum. Known positive sera were included in all tests.

EXPERIMENTAL

The Antibody Response Following Experimental Exposure to Mumps Virus.—In order to study the development of antibodies to the two antigens in the course of infection with mumps virus, it was felt that experimental exposure of man to this agent would yield more dependable data, since the time of expo-

² We are indebted to Sharp and Dohme, Inc., for a generous supply of this material.

sure would be accurately known. Sera of such experimental cases were available from studies for other purposes which will be reported separately (9). Fig. 1 demonstrates graphically the results of complement fixation tests with the V and S antigens and sera taken from a number of representative cases at varying intervals after experimental exposure to an oral spray of three different virus preparations. Cases F.G. and G.C. had been exposed, with other individuals, to 5th passage amniotic fluid infected with a strain of mumps virus (J.P.), which had been isolated directly in eggs by the amniotic route from the spinal fluid of a patient with meningoencephalitis (10). F.G. and others not shown in the figure developed parotitis on about the 18th day after exposure; G.C. failed to do so and remained apparently well. On comparing the sero-



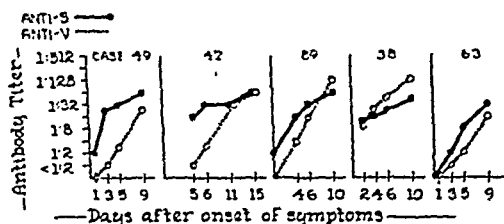
TEXT-FIG. 1. Development of antibodies following experimental exposure to mumps virus.

logical results, it can be seen that both individuals developed antibodies to about the same extent, although only one became clinically ill. This result appears comparable to subclinical infections encountered under epidemic conditions (2, 4, 11). In both cases, antibodies to S antigen appeared earlier and reached high levels before anti-V commenced to rise. It should also be noted that anti-S had already reached a high titer at the time parotitis became manifest.

Cases E.C., J.J., and C.H. were exposed to 16th passage allantoic fluid of the strain of virus received from Dr. Enders; *i. e.*, the same agent which was used for the preparation of the antigens. These subjects, as well as several others not shown in the figure, failed to develop clinical signs of mumps. This strain, therefore, may be considered attenuated in regard to its pathogenicity for man. It can be seen that anti-S developed after about the same interval as in the

cases exposed to the virulent strain of virus. The lag in the development of anti-V was quite marked in case E.C., and patient J.J. failed altogether to develop a significant level of this antibody. The serological response of patient C.H., on the other hand, was practically indistinguishable from that of the cases F.G., and G.C., exposed to the virulent virus. Finally, the sixth patient, L.H., serves as an example of several cases exposed to infected monkey parotid gland, supplied by Dr. J. F. Enders. In this patient, parotitis developed on the 20th day after exposure, at a time when both these antibodies were as yet low in titer. Again anti-S appeared somewhat before anti-V. The difference between these two antibodies might have been more strikingly demonstrable if sera had been available between the 22nd and 32nd days after exposure.

The Antibody Response Following Natural Infection.—Sera from cases of the natural disease during the epidemic of 1946–47 could be collected only after appearance of symptoms. The date of onset was supplied by the parents and its accuracy was possibly influenced by their vigilance. The serological data



TEXT-FIG. 2. Development of antibodies in epidemic cases of mumps.

obtained in many of these cases demonstrate that the antibody response in the natural disease is similar to that encountered after experimental exposure to the virus, as shown in Fig. 2 (cases 47, 49, and 89). The antibodies against S usually rise prior to those against V. In some of the cases, the first serum may have been taken too late to show this difference clearly, as in case 38, but the slope of the respective antibody curves indicates that anti-S may have been exceeding anti-V prior to the date of the first bleeding. Finally, case 63 is included to show that in some patients anti-V may develop simultaneously with anti-S or even prior to the latter.

These data demonstrate that the reactivity of sera taken in the first few days of illness frequently is restricted to interaction with the S antigen. As a rule, this reaction leads to complete fixation of complement in all tubes up to the second last effective dilution of serum, the last giving only partial fixation. On occasions, however, the reactions with S antigen show marked zoning, and only partial fixation of complement, over a wide range of serum dilutions. This phenomenon has been observed previously by Enders, Cohen, and Kane (2), with antigens prepared from infected parotid glands of monkeys. Examples of

this activity are listed in Table II. Sera taken later in the disease always show the usual complete fixation of complement over a series of dilutions and partial fixation in only one or two further tubes. This reactivity of early sera is considered significant but its interpretation is not yet clear.

The frequency with which these antibody relationships are encountered may be gleaned from Fig. 3. In this figure results are collected of complement

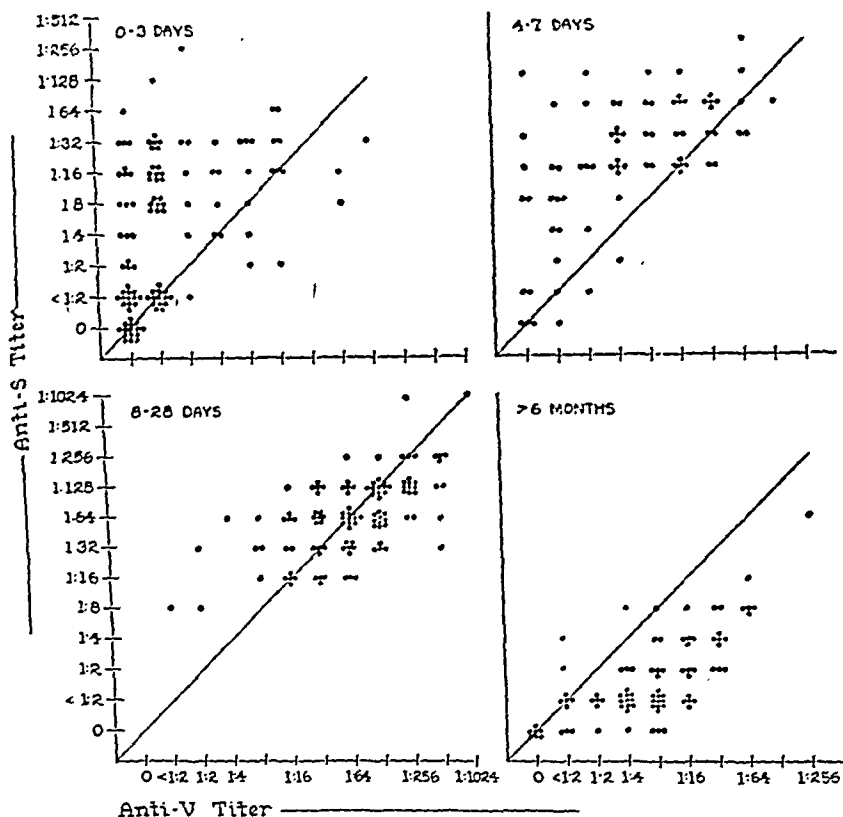
TABLE II

Cases of Mumps Showing Zoning and Partial Fixation of Complement with S Antigen in the First Days after Onset

Institution	Patient No.	Onset of disease	Time after onset	S antigen								V antigen
				Dilution of serum								
				1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Serum titer
		1947	days									
D	77	3/9	2		st	wk	wk	ac	(c)			0
			19		0	0	0	(0)	wk			32
D	55	2/17	1		c	st	tr	wk	c			0
			28		0	0	0	0	(0)	c		256
H	131	4/11	4	wk	tr	0	0	0	0			8
			7	0	0	0	0	0	0			32
H	154	4/26	2	ac	st	wk	ac	(c)	c			0
			4	st	wk	tr	(0)	tr	ac			0
			6		(0)	0	0	(0)	wk			4
H	153	4/25	1	wk	tr	(0)	(0)	(0)	st			0
			3	0	0	0	0	0	0			<2
S	189	4/12	0	wk	wk	st	st	ac				<2
			11			0	0	0	tr	wk		64
S	168	3/30	4		tr	tr	tr	tr	wk	ac		8
			19				0	0	0	0	ac	256

fixation tests with several hundred sera. Each dot represents one serum showing the titer of antibodies against S on the ordinate and that against V on the abscissa. The sera are grouped according to the time they were taken after the reported day of onset of mumps. It can be seen that in the 1st week of illness, most of the dots lie above the diagonal line, indicating that anti-S exceeds anti-V. In the first 3 days some of the sera have no antibodies against either S or V; many have no or low anti-V but already high anti-S antibodies; some are high in both, and in only a few the anti-V antibodies exceed the anti-S.

From 4 to 7 days after onset, only very few of the patients fail to reveal significant levels of antibodies against both antigens. The incidence of cases with high titers against S but low levels of antibodies against V has decreased. From 8 to 28 days after onset practically all cases possess high levels of antibodies against V and S. Of the sera charted almost all had reached titers against the



TEXT-FIG. 3. Correlation between antibody levels against S and V antigens at various times after onset of mumps.

S antigen of 1:16 or higher, and most of them attained similar levels when tested with the V antigen. Thereafter the antibodies begin to decrease in titer, the S antibodies usually at a somewhat faster rate than those reacting with V. If sera are analyzed, which were collected 6 months to several years after infection with mumps virus, it can be seen that in practically all of them anti-V exceeds anti-S. The latter antibody may be low or absent in sera which show relatively high levels of anti-V. The sera of certain patients analyzed several years after an established infection with mumps virus show no detectable antibodies against either antigen.

In a comparison of results from uncomplicated cases of mumps with those of patients showing complications, such as meningoencephalitis, orchitis, pancreatitis, and others, no significant qualitative differences became apparent but the levels of antibodies tended to be higher in such patients. This difference was about twofold when the geometric mean antibody levels were calculated for the two groups. Since only few complications were encountered during the mumps epidemic of 1946-47, further observations are required to ascertain whether or not there is a significant relationship between the time of appearance of antibodies and their concentration on the one hand, and the development of complications on the other.

The Rôle of the V and S Antigens in the Diagnosis of Mumps.—A summary of the data presented, thus far, provides a diagnostic scheme as shown in Table III. If the serum of a patient has a high titer against the S antigen (1:16 or higher) and few or no antibodies against V, the present illness is probably

TABLE III
Tentative Interpretation of Serological Results

Anti-S titer	Anti-V titer	Interpretation
High	Low or absent	First days of disease
High	High	Recent apparent or inapparent infection, or current mumps of several days' duration
Low or absent	Positive to varying extent	Long past apparent or inapparent infection

caused by mumps virus. High levels of both antibodies indicate a recent or a current attack of several days' standing. Little or no anti-S, and distinct levels of anti-V (1:2 or higher) are considered signs of long past infection. The table provides certain criteria for diagnosis which must be interpreted in the light of the individual patient.

It has already been pointed out that the order of events in the first days of illness does not always conform to the presented picture, but it does so sufficiently often to permit the early diagnosis of certain clinical manifestations, such as mumps meningoencephalitis in the absence of preceding or concurrent parotitis. Table IV shows serological data obtained in 12 cases of mumps meningoencephalitis in which parotitis was absent or developed at a later date (case L.H.).³ In 8 of the 12 cases, the finding of high anti-S and low anti-V levels provided a serological diagnosis of mumps early after onset. Six of these 8 cases from whom a second serum specimen was obtained at a later date showed

³ The sera of 2 of these cases (L.M., and M.D.) were submitted by Dr. A. J. Rhodes, of the Connaught Medical Research Laboratories, Toronto, Canada.

TABLE IV

Early Diagnosis of Mumps MeningoEncephalitis without Parotitis by Complement Fixation Tests with S and V Antigens

Patient	Time after onset <i>days</i>	Serum titer rr.		Remarks
		S antigen	V antigen	
H.C.	8	256	8	
	15	512	32	
L.H.	6	64	4	Parotitis on 14th day
	13	64	64	
C.B.	4	32	<4	
	18	64	64	
M.D.	2	64	4	
	14	32	128	
J.P.	2	16	<2	No second serum, virus isolated from spinal fluid
D.L.	2	16	<2	
	15	64	256	
Nys.	2	256	2	No second serum
J.S.	6	64	<4	
	19	128	64	
Ahr.	4	4	<2	
	18	16	16	
Mar.	3	4	2	
	15	16	64	
A.P.	2	4	4	Virus isolated from spinal fluid
	6	4	8	
	10	8	32	
	17	16	128	
Wri.	2	<2	4	
	12	128+	128+	

subsequent rises particularly in anti-V. From the spinal fluid of one of the 2 patients from whom a second serum could not be obtained, mumps virus was isolated. This confirmed the result of the complement fixation test (10). The

serological diagnosis of the remaining 4 cases listed in Table IV depended, as in the studies of Kane and Enders (3), on the demonstration of rises in antibodies over a period of 1 week or longer. Significant rises in antibodies were measured with both antigens. In several additional patients with meningo-encephalitis, the first available sera were taken rather late in the disease and yielded high titers against both V and S antigens. In these, no rises in antibodies could be measured and thus definite proof was not obtained that the neurological signs were a result of infection with mumps virus.

TABLE V
Antibody Response Following Subcutaneous Vaccination or Skin Testing

Case No.	Vaccination				Case No.	Skin testing			
	Anti-V		Anti-S			Anti-V		Anti-S	
	A	B	A	B		A	B	A	B
1	0	0	0	0	1	0	0	0	0
2	0	0	0	0	2	0	0	0	0
3	0	6	0	3	3	0	8	0	0
4	<2	48	<2	12	4	0	32	0	0
5	<2	48	0	32	5	0	32	0	8
6	<2	24	<2	4	6	<2	4	<2	4
7	<2	24	<2	2	7	<2	16	<2	2
8	<2	128	0	8	8	<2	32	4	8
9	2	8	0	<2	9	<2	32	<2	4
10	2	96	<2	6	10	<2	64	0	4
11	2	128	0	24	11	2	32	<2	8
12	4	48	<2	16	12	2	16	0	0
13	16	96	3	8	13	4	16	0	0
					14	4	32	0	0

A = serum taken before vaccination or skin test.

B = serum taken 2 weeks after vaccination or skin test.

The Antibody Response Following Vaccination and Skin Testing.—It has been shown by others that both vaccination of monkeys and man with inactivated mumps virus (12-14) and skin tests in man (2, 15) performed with mumps antigen, caused the formation of complement fixing antibodies. These observations were confirmed in a study of a number of cases of each type. Centrifugally concentrated virus served as vaccine and infected allantoic fluid as skin test material. The virus in both instances was inactivated by ultraviolet irradiation. Table V summarizes the data selected from representative groups which show the variability of the response. The results obtained following the two types of stimuli were very similar. As a rule, the antibodies against V developed to a distinctly higher titer than those against S. In the majority of

these cases a restimulation of antibodies is indicated by the fact that low levels of anti-V were found in many of the sera taken before the skin test or before vaccination, a finding compatible with past apparent or inapparent infection. Among those cases whose sera did not react with V antigen prior to vaccination or skin testing, some responded with the formation of antibody, others did not. Whether those who responded had lost their antibodies in the years following infection, and whether their reaction must therefore be classified as restimulation, or whether the response represents antibody formation *de novo*, cannot be stated at present.

Non-Specific Reactions.—The reactions obtained with these antigens in the majority of the sera studied appeared specific in that the sera failed to react with control antigens. However, sera were encountered, on occasion, which gave positive complement fixation tests with suspensions of normal allantoic membrane. These occurred in spite of heating to 60°C. for 20 minutes. A second heating to 60°C. (2) removed some, but not all, of the non-specific reaction. This reactivity with control antigen affected particularly the interpretation of the reactions with S antigen and not so much those with V antigen. Absorption of such sera with particulate components of normal allantoic sacs sedimentable at 20,000 R.P.M., removed this reactivity and uncovered the specific reaction with the S preparation. Table VI demonstrates several such experiments. As can be seen, the three serum specimens obtained from case 64 all reacted to about the same extent with N antigen. After absorption this reactivity was lost, whereas the reaction with the S preparation, although reduced by the absorption, showed the expected characteristic rise in titer during the first few days of parotitis. The reaction with V was not measurably affected by the absorption, nor was the Wassermann reaction. The latter was apparently a false positive, inasmuch as it became negative during convalescence (16). Case 67 gave essentially similar results except that a true positive Wassermann test was encountered in this patient. Case 46 is included to show that absorption with normal membrane particles does not affect the titer against S antigen in sera which fail to show a reaction with N.

The absorption experiments seemed to indicate that antibodies to normal chick components may occur in man on occasion and that Wassermann antigen is not involved in this reaction. To elucidate this question further, sera were obtained from 6 luetic patients with no recent history of mumps. None of these reacted with the normal membrane antigen and 4 were free of specific mumps antibodies. These data suggested, on the other hand, that heterophile antibodies, possibly related to Forssman antibodies, might be responsible for some of the non-specific reactions. This possibility was further supported by the observations that the sensitized sheep cells in the complement fixation test were agglutinated by some of the sera in the lower dilutions. Absorption of such sera with washed sheep erythrocytes frequently removed the non-specific

TABLE VI

Removal of Non-Specific Reactions by Absorption with Particulate Components Derived from Normal Allantoic Membranes

Case No.	History	Date of bleeding	Treatment of serum	Antibody titers vs.			Wassermann reaction
				N antigen	S antigen	V antigen	
64	Pansinusitis 3/9 Parotitis 3/13	1947	—	1:32	1:32	1:16	+
		3/13	Absorbed	<1:4	1:8	1:16	+
		3/15	—	1:32	1:32	1:64	
		3/15	Absorbed	—	1:8	1:64	
67	Lues. Skin test for mumps 3/15	3/15	—	1:32	1:64	1:128	
		3/15	Absorbed	—	1:32	1:128	
		3/31	—	1:16	1:16	1:4	+
		3/31	Absorbed	1:2	1:4	1:4	+
46	Parotitis 3/3	3/4	—	1:16	1:32	1:128	+
		3/4	Absorbed	<1:4	1:16	1:128	+
		3/8	—	<1:2	1:32	<1:2	
		3/8	Absorbed	—	1:32	<1:2	
46	Parotitis 3/3	3/8	—	—	1:128	1:24	—
		3/8	Absorbed	—	1:128	1:32	—
		3/12	—	—	1:128	1:128	
		3/12	Absorbed	—	1:128	1:128	

TABLE VII

Removal of Non-Specific Reactions by Absorption with Sheep Erythrocytes

Case No.	History	Date of bleeding	Treatment of serum	Antibody titers vs.		
				N antigen	S antigen	V antigen
D4	Positive (parotitis 3/7/47)	1947	—	1:64	1:64	1:4
		3/7	—	<1:4	<1:4	0
		3/21	—	1:32	1:64	1:64
		3/21	Absorbed	<1:2	1:32	1:64
D7	Negative	3/3	—	1:8	1:8	
		3/3	Absorbed	0	0	
51	Positive (parotitis 2/28/47)	3/17	—	0	1:64	1:32
		3/17	Absorbed	0	1:64	1:32

reactivity, whereas absorption of specific mumps convalescent sera revealed that the mumps antibodies were not affected by such procedures. Table VII shows some examples of this kind.

DISCUSSION

The data presented show that sera obtained from human beings at various stages of infection with the virus of mumps may differ distinctly in their content of antibodies against the two antigens, the virus particle (V) and the soluble antigen (S). In the early days of disease, anti-S antibody may reach high titers, whereas anti-V may still be low or absent. This has permitted the serological recognition of an infection with mumps virus, on occasion, on the 1st or 2nd day of illness. This presumptive test has been confirmed in all instances studied by the demonstration of rises in antibodies in subsequent sera. It was found of great assistance in the early diagnosis of several cases of mumps meningoencephalitis in the absence of parotitis.

For determination of resistance to mumps, use of the V antigen alone appears sufficient. Many more individuals, who experienced either apparent or inapparent infections at some time in the past, will react with V than with S. The rôle played by anti-V in the resistance of an individual to mumps has not been definitely established. However, as has been shown (11), in a small group of children, no cases of mumps occurred among those giving significant reactions with V prior to an epidemic. On the other hand, not all of the children whose sera failed to react with V developed clinical or subclinical infections. This latter observation may depend upon several possible factors: (a) the intimacy of exposure may vary; (b) amounts of anti-V too small to measure may, nevertheless, give protection, and (c) an antibody different from that measured by the described complement fixation technic may be responsible for resistance.

A comparison of the data published by Enders and his coworkers (1-4), with those presented above, indicates that the antigen in suspensions of infected parotid glands of the monkey is dominantly of the S type. This suggestion is based particularly upon the fact that the marked zoning and the incomplete fixation of complement with occasional sera of the early acute phase which have been observed using monkey antigen were also found to occur employing the S antigen of the chick embryo, but not the V preparation. This suggestion is in line too with the finding of high antibody levels against monkey parotid antigen in some other patients on the first few days of disease. The monkey antigen, as prepared, contains virus particles. Whether the amount of it is sufficient to be reactive in the complement fixation reaction cannot be stated until strict comparisons are made of the various preparations of antigen with suitable immune sera.

The serological response obtained under conditions of experimental infection with a human pathogenic strain of mumps virus appeared to be similar to that

of the epidemic illness in every respect studied. Furthermore, the exposure of human beings to "attenuated" mumps virus gave, in most instances, antibody levels against V and S antigens comparable to those seen in uncomplicated cases of the natural disease. These data seem to indicate, therefore, that such a procedure may lead to an enduring immunity, a suggestion which has already been explored (17) and which will be investigated further.

Intradermal or subcutaneous injection of inactivated mumps virus has been shown in the past to stimulate formation of complement-fixing antibodies (2, 12-15). The present studies revealed that frequently only anti-V was formed. If anti-S also developed, its titer constituted, as a rule, only a fraction of that measured against V. Antibody formation in these cases was noted particularly if the treated individual already possessed some antibodies, whereas only part of those with negative complement fixation tests responded to such stimuli. It is possible that in these instances too the rise in antibodies was the result of restimulation by small quantities of antigen, whereas for the *de novo* formation of antibodies, possibly more concentrated preparations of virus may be needed. These relationships call for further analysis.

SUMMARY

Human sera taken at various stages of mumps have been analyzed in regard to their reactivity with two serologically distinct complement fixation antigens which were derived from the infected chick embryo. Antibodies to the soluble or S antigen appear earlier in the disease and, as a rule, reach high levels before antibodies against the virus-bound or V antigen commence to rise. In early convalescence, both antibodies reach high levels. Subsequently antibodies against the S antigen decrease usually at a faster rate than those against V, so that after a period of several years, frequently only anti-V may be left.

These findings were found helpful in diagnostic procedures. The use of both the V and S antigens has permitted the early diagnosis of manifestations of mumps in the absence of parotitis, such as meningoencephalitis, since the finding of high levels of anti-S and of low titers of, or no, anti-V is considered diagnostically significant for the first few days of illness. For the determination of resistance the use of the V antigen appears more useful.

Following vaccination or skin testing, antibodies against both antigens may develop; those against V increase more regularly and to higher titers than those against S.

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THE EFFECT OF NUCLEIC ACIDS AND OF CARBOHYDRATES ON THE FORMATION OF STREPTOLYSIN*

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Yeast nucleic acid has been shown by Okamoto (1) to induce the formation of a potent hemolysin in cultures of *Streptococcus pyogenes*. As reported by Okamoto, and confirmed in the present paper, the effect of nucleic acid is readily demonstrable since the hemolytic activity of filtrates or supernates of nucleic acid-broth cultures is at least 20 to 100 times greater than the activity of cultures prepared in broth to which no nucleic acid has been added. On blood agar containing yeast nucleic acid, the effect is manifest by the appearance of very large zones of beta hemolysis around the colonies.

The nucleic acid effect has been investigated further in order to define the conditions necessary for producing the hemolysin on a large scale and in a manner which will facilitate its purification. In addition, although some of the properties of the nucleic acid-induced hemolysin have been described by Itô (2), the relationship of this hemolysin to the better characterized streptococcal hemolysins, streptolysin O and streptolysin S, requires clarification. Finally, an opportunity is afforded of studying an almost unknown biological effect of nucleic acid.

Methods, Materials, and Terminology

Preparation of Peptone-Infusion Broth.—Fresh beef hearts, freed of gross fat, were ground and then mixed well with tap water, 1 liter of water for each pound of ground heart. After being brought to 85°C. and infused at that temperature for 45 minutes, the mixture was filtered while hot, through paper. To each liter of infusion was added 10 gm. peptone (neo-peptone Difco) and 5 gm. reagent sodium chloride. After the ingredients were completely dissolved by bringing to a boil, the pH was brought to 7.9, the medium was boiled 1 to 2 minutes, and then filtered immediately through paper. The medium was sterilized in the autoclave at 121°C. for 15 to 30 minutes. Immediately before inoculating, a freshly prepared solution of sodium thioglycollate was added to give a final concentration of 0.01 per cent.

Culture Technique.—A 6 to 8 hour peptone-infusion broth culture of *Streptococcus pyogenes*, strain C203S, was distributed in 0.5 cc. amounts among a large number of sterile tubes, and then rapidly frozen solid in an alcohol-carbon dioxide freezing mixture. The tubes were stored in a dry-ice chest until needed. When required, a tube was thawed at room temperature, the contents diluted 10 times in sterile saline, and 0.1 cc. inoculated into each 10 cc. of

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culture medium. Cultures were 10 cc. in volume, and were contained in 16×150 mm. culture tubes. They were incubated at 37°C . for 18 to 24 hours. Unless otherwise indicated, the strain of streptococcus employed was C203S.

Estimation of Hemolytic Activity.—The method is the same as that employed in previous studies (3). The hemolytic unit is the amount of hemolysin which will lyse half the erythrocytes (human) contained in 2 cc. of phosphate-buffered saline, pH 7, in 30 minutes at 37°C . It has been observed that the stock suspension of washed erythrocytes slowly increases in sensitivity to the nucleic acid-induced hemolysin even though the suspension is stored at $3-5^{\circ}\text{C}$. An erythrocyte suspension which is kept in the cold for 5 days is lysed by about half as much nucleic acid-induced streptolysin as that required to lyse suspensions prepared from fresh cells. The age of the erythrocyte suspension must therefore be taken into account when the same suspension is used for experiments performed over a period of several days.

Preparation of Streptococcal Nucleic Acid.—*Streptococcus pyogenes*, strain C203S, was grown in 30 liters of casein hydrolysate medium according to the method described earlier (4). After centrifugation of the cultures, the cells were washed in saline, and dried *in vacuo* from the frozen state. Yield: 25 gm.

8 gm. dried cocci was ground in a mill of quarter-inch steel balls. The mill, one designed by Swift and Hirst (5), was charged with 2 gm. of cocci at a time, and operated for 20 hours at room temperature for each charge. Each 2 gm. of ground cocci was washed from the mill with portions of demineralized water totalling 100 cc. The pooled mill-washings were centrifuged, the insoluble "cell fragments" were washed once in water, and the washing added to the supernatant aqueous extract. The aqueous extract contained 70 per cent of the total phosphorus present in the intact cocci. Nucleic acid, chiefly of the ribose type, was isolated from the acid-precipitated nucleoprotein contained in the aqueous extract, according to the procedure of Sevag, Lackman, and Smolens (6). Yield: 0.82 gm.

Analytical Methods.—Total phosphorus was determined by the method of Lohmann and Jendrassick (7). Nitrogen was estimated by a modified micro Kjeldahl procedure. Purine ribose was estimated by the orcinol-pentose reaction, employing conditions essentially the same as those of Mejbbaum (8). Under these conditions, the reaction measures purine ribose to the exclusion of pyrimidine ribose (9).

Terminology.—The terms, ribonucleic acid and desoxyribonucleic acid, are employed with the knowledge that the pentose and desoxypentose of nucleic acids other than those of yeast and thymus, in most cases, have not been identified.

EXPERIMENTAL

I. Nucleic Acids

Streptolysin Production as a Function of Concentration of Yeast Sodium Nucleate.—A series of tubes of broth containing different concentrations of commercial yeast sodium nucleate were inoculated and incubated at 37°C . After 18 hours, the hemolytic activity of the cultures was measured and plotted as a function of sodium nucleate concentration. As can be seen in Fig. 1, nucleate concentrations of less than 1 mg. per cc. had little effect, while concentrations between 1 and 4 mg. per cc. caused increasing amounts of hemolysin to appear. In this experiment, concentrations greater than 4 mg. per cc. caused no further increase in the amount of hemolysin. The slope of the linear portion of the curve, and of curves obtained in similar experiments, showed that on the average, doubling the concentration of sodium nucleate

approximately tripled the hemolytic activity. The maximum hemolytic activity varied with different lots of broth, from 2000 hemolytic units per cc., as in the present experiment, to 4000 hemolytic units per cc. in others.

When streptococci which had grown in nucleic acid-broth were transferred to plain broth, the amount of streptolysin formed was the same as that usually found in plain broth, namely 30 to 100 hemolytic units per cc. It is evident, therefore, that nucleic acid does not induce a permanent change in the capacity of streptococci to form hemolysin. Maintenance of the effect in serial cultures requires the continued presence of nucleic acid.

Sodium nucleate had no effect on the amount of bacterial growth as measured turbidimetrically. The final pH of cultures containing high concentrations of

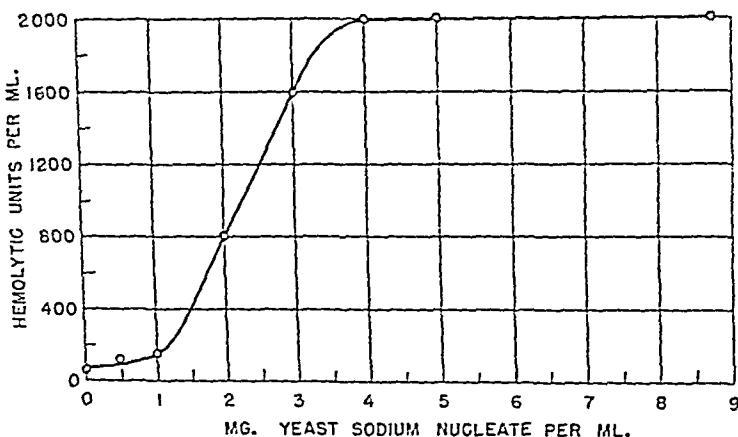


FIG. 1. Hemolytic titer of broth cultures as a function of concentration of yeast sodium nucleate.

sodium nucleate did not differ by more than 0.17 unit from that of cultures grown in the absence of sodium nucleate.

Probable Identity of Nucleic Acid-Induced Streptolysin with Streptolysin S.—Streptococci of the Lancefield group A (*Streptococcus pyogenes*) have been shown by Todd (10) to produce two distinct hemolysins designated streptolysin O and streptolysin S. Either or both hemolysins are formed *in vitro* depending upon the strain, the culture medium, and the conditions of incubation (11). The properties of these hemolysins are described in the papers of Todd (10) and Herbert and Todd (11, 12).

Study of the nucleic acid-induced hemolysin has shown that it is not activated by reducing agents nor is it neutralized by appropriately diluted sera containing antistreptolysin O. It cannot be streptolysin O, therefore. The nucleic acid-induced hemolysin has the following additional properties: (a) Unlike certain other hemolysins, including streptolysin O, it is not inhibited

by cholesterol (2, 13). (b) It is irreversibly inactivated by dilute acid and by brief heating. (c) Its production in infusion broth is suppressed by glucose (14). (d) Initiation of hemolysis is preceded by a relatively long latent period (13). In these respects, the nucleic acid-induced streptolysin is identical with streptolysin S. There is, moreover, no evidence that the two hemolysins are not the same.

If the nucleic acid-induced hemolysin and streptolysin S are identical, it follows that yeast nucleic acid should cause increased hemolysin production in strains known to produce streptolysin S. It follows, also, that yeast nucleic acid should not increase the formation of hemolysin by strains which produce only streptolysin O. A series of strains of group A streptococci were investi-

TABLE I
Nucleic Acid Effect in Relation to Strain of Group A Streptococcus

Strain	Produces streptolysin S	Produces streptolysin O	Hemolytic titer of plain broth culture			Hemolytic titer of broth culture containing 0.2 mg. ribonuclease-resistant fraction of yeast nucleic acid per cc.			
			Hrs. of incubation at 37°C.			Hrs. of incubation at 37°C.			
			23	48	96	23	48	96	168
C203S	+	+	<100	<100	<100	2650	2260	2000	1200
1685M	+	?	<100	<100	<100	100	600	2000	600
Blackmore	+	—	<100	<100	<100	2000	2000	2650	750
Lang	+	—	<100	<100	<100	2000	2000	2650	750
C203U	—	+	<100	<100	<100	<100	<100	<100	<100
Wilson 868	—	+	<100	<100	<100	<100	<100	<100	<100
Hodder 872	—	+	<100	<100	<100	<100	<100	<100	<100
Tucker 873	—	+	<100	<100	<100	<100	<100	<100	<100

gated from the point of view of the nucleic acid effect. The strains studied included several (Blackmore, Lang, Wilson 868, Hodder 872, and Tucker 873) which had been previously analyzed by Colebrook *et al.* (15) and by Herbert and Todd (11) for their capacity to produce streptolysins O and S.

The results, presented in Table I, show that the nucleic acid effect is readily demonstrable when strains known to produce streptolysin S are employed, and that a comparable effect is not observed in strains which lack this capacity. These results constitute strong evidence for the identity of nucleic acid-induced streptolysin with streptolysin S, particularly when considered together with the fact that the properties of the two hemolysins do not allow them to be differentiated.

Streptolysin-Inducing Activity of Various Nucleic Acid Preparations.—The usual methods of purifying nucleic acids did not alter appreciably the activity of commercial yeast nucleic acid. These methods included deproteinization

with chloroform and amyl alcohol (6), precipitation with glacial acetic acid (16), ethanol or barium (17), dialysis, and combinations of these procedures. In

TABLE II

Streptolysin-Inducing Activity of Different Preparations of Ribonucleic and Desoxyribonucleic Acids. Effect of Ribonuclease on Streptolysin-Inducing Activity

Type and source of nucleic acid	Method of preparation	Amount required to induce formation of 1000 hemolytic units
		mg.
1. Ribonucleic acid, yeast	Commercial (Ma)	4.0
2. Ribonucleic acid, yeast	Commercial (Pf)	2.3
3. Ribonucleic acid, yeast	Commercial (Sci)	5.0
4. Ribonucleic acid, yeast	Commercial (Sc2)	4.0
5. Ribonucleic acid, yeast	Commercial (El)	6.0
A 6. Ribonucleic acid, yeast	Commercial (E2)	6.0
7. Ribonucleic acid, beef liver	Mirsky and Pollister (32), modified	0.6
8. Ribonucleic acid, wheat germ	Clarke and Schryver (33), modified	2.5
9. Desoxyribonucleic acid, thymus*	Hammarsten (34)	>10.0†
10. Desoxyribonucleic acid, thymus (?)	Commercial	>10.0§
11. Desoxyribonucleic acid, human placenta	Hammarsten, modified	>10.0
12. No. 3 treated with ribonuclease		0.5
13. No. 7 treated with ribonuclease		0.6
14. No. 8 treated with ribonuclease		0.7
15. Nucleic acid, streptococcal, strain 1685M, treated with ribonuclease	Sevag, Lackman, and Smolens	1.0
B 16. Nucleic acid, streptococcal, ¶ strain C203S, treated with ribonuclease	(See Methods)	0.8
17. Ribonucleic acid, tobacco mosaic virus** treated with ribonuclease		>2.0

* Kindly supplied by Dr. Milton Levy, New York.

† 5 mg. per cc. induced formation of 150 hemolytic units per cc.; control: 60 hemolytic units per cc.

§ 5 mg. per cc. induced formation of 125 hemolytic units per cc.; control: 125 hemolytic units per cc.

|| Kindly supplied by Dr. M. G. Sevag, Philadelphia. Chiefly ribonucleic acid, but stated to contain 10 to 30 per cent desoxyribonucleic acid.

¶ Chiefly ribonucleic acid.

** Kindly supplied by Dr. H. S. Loring of Stanford University, California.

view of these findings, it seemed worth while to investigate the activity of nucleic acids from different sources.

The sodium salts of eight preparations of ribonucleic acid and three of

desoxyribonucleic acid were tested for streptolysin-inducing activity. The sources of these preparations and the quantity of each required to cause the formation of 1000 hemolytic units are shown in Table II, A. All the preparations of ribonucleic acid including those from liver and from wheat as well as those from yeast were active. Not all of the preparations, however, were equally effective in causing streptolysin formation, and it is particularly notable that the ribonucleic acid from liver and that from wheat were more active than most of the preparations derived from yeast. The three specimens of desoxyribonucleic acid contrasted strongly with ribonucleic acid in failing to stimulate streptolysin formation.

Specificity of Ribonucleic Acid in Inducing Streptolysin Formation.—A number of naturally occurring materials were tested in order to determine whether any of them produced the same effect as ribonucleic acid when added to broth. These included yeast extract (20 mg. per cc.), Witte's peptone (100 mg. per cc.) acid hydrolysate of casein (0.6 per cent by volume), crystalline hemoglobin (2 mg. per cc.), horse serum (10 per cent by volume), protamine (1 γ per cc.), lecithin 3.5 mg. per cc.), brain phosphatides (4.3 mg. per cc.), lipovitellin (1 mg. per cc.), glycogen (0.5 mg. per cc.), and a considerable number of other polysaccharides. With the exception of lecithin and rabbit serum, none of these materials was active. Lecithin and rabbit serum caused slightly greater hemolysin formation than that which occurred in broth to which nothing had been added. The effect of serum and lecithin was less than one-twentieth of that of comparable concentrations of ribonucleic acid.

Adenosine triphosphate and diphosphopyridine nucleotide in concentrations of 0.5 and 1.0 mg. per cc., respectively, were found to be inactive. In addition, the following substances were found to have no activity: yeast adenylic and guanylic acids (0.4 mg. per cc.); adenosine and guanosine (0.4 mg. per cc.); adenine, guanine, uracil, and cytosine (0.1 mg. per cc.); *d*-ribose (0.4 mg. per cc.). In one experiment, however, adenosine appeared to be active in stimulating streptolysin formation. Subsequent experiments failed to confirm this finding.

The following hydrolysates of yeast nucleic acid were prepared:

(a) 5 gm. yeast nucleic acid was refluxed with 50 cc. 2 per cent sulfuric acid for 2 hours. The mixture was neutralized with sodium hydroxide. (b) 2.5 gm. of yeast nucleic acid was heated with 25 cc. of 2.5 per cent ammonium hydroxide in a sealed tube for 60 minutes at 115°C. After removing a gelatinous precipitate, sodium hydroxide was added, and ammonia removed under reduced pressure in a stream of air. (c) 2 gm. yeast nucleic acid was allowed to stand at 28°C. in 0.5 N sodium hydroxide. After 3 hours, the pH was adjusted to 7.5.

Each of the three hydrolysates was tested for streptolysin-inducing activity; none was found to be active.

Effect of Ribonuclease on Activity of Ribonucleic Acid.—Since the streptolysin-

inducing activity of yeast ribonucleic acid was found to be destroyed by either acid or alkaline hydrolysis, it was of interest to study the effect of enzymatic degradation. For this purpose, crystalline ribonuclease prepared from beef pancreas (16) was employed.

To 100 mg. sodium nucleate (yeast) dissolved in 4 cc. water was added 1 cc. 0.1 per cent solution of ribonuclease. After adjusting the pH to 7.6 and allowing digestion to proceed for 4 hours at room temperature, the solution was passed through a Seitz filter and tested for streptolysin-inducing activity. The results, together with those obtained on testing sodium nucleate which had not been treated with ribonuclease, are shown in Table III.

TABLE III
Activation of Yeast Ribonucleic Acid by Ribonuclease

Material added to broth	Final concentration mg./cc.	Hemolytic units per cc. culture
Sodium nucleate	4.0	500
	2.0	125
	1.0	<30
	0.5	<30
Sodium nucleate plus ribonuclease	0.5	1000
	0.25	400
None	0.0	<30
Ribonuclease	0.05	<30

It can be seen that as little as 0.5 mg. of digested nucleic acid stimulated the formation of 1000 hemolytic units while 8 times as much of the undigested nucleic acid stimulated the formation of only 500 hemolytic units. Numerous experiments, in which time and concentrations were varied, showed that, on the average, ribonuclease digestion caused a tenfold activation of yeast ribonucleic acid. Ribonuclease itself was found not to affect hemolysin production. Since the ribonuclease was known to be contaminated with trypsin and since its activating effect could conceivably be attributed to the latter, nucleic acid was incubated with a solution of crystalline trypsin, and then tested for streptolysin-inducing activity. No change in activity occurred.

When ribonucleic acid derived from wheat germ was treated with ribonuclease, its activity increased $3\frac{1}{2}$ times (Table II, B). Liver ribonucleic acid, however, showed no change in activity when treated with the enzyme. It is notable that the activity of enzyme-treated ribonucleic acid is approximately the same regardless of whether the ribonucleic acid derives from yeast, mammalian liver, or wheat germ (Table II, B).

Three additional preparations of nucleic acid were tested for streptolysin-inducing activity. Two of these were derived from group A streptococci, strains 1685M and C203S, respectively, while the third was ribonucleic acid of tobacco mosaic virus. Since these materials were available only in very small quantities, they were treated with ribonuclease prior to testing, on the assumption that activity could be detected if it were of the same order as that of enzyme-treated ribonucleic acid of wheat, liver, or yeast. As can be seen in Table II, B, nucleic acid from each of the two streptococcal strains was active, while that of tobacco mosaic virus was not. It is evident, therefore, that the nucleic acid derived from tobacco mosaic virus differs from all the other ribonucleic acid preparations in failing to cause lysin formation.

TABLE IV

Stability of Streptolysin-Inducing Activity of Ribonuclease-Treated Yeast Ribonucleic Acid

pH of solution	Treatment	Residual streptolysin-inducing activity compared to that (1.00) of solution heated at 100°C. for 10 min. at pH 7.1
2.7	Boiling water bath for 10 min.	0.25
4.1	Boiling water bath for 10 min.	0.50
7.1	Boiling water bath for 10 min.	1.00
7.5	Autoclave at 120°C. for 20 min.	0.65
8.5	Boiling water bath for 10 min.	0.27
9.5	Boiling water bath for 10 min.	0.17

In view of the effect of ribonuclease on the activity of yeast nucleic acid, there arose the question of whether desoxyribonucleic acid might not become active when treated enzymatically. Experiments in which desoxyribonucleic acid was treated with desoxyribonuclease, or with ribonuclease, showed that desoxyribonucleic acid is not activated by either of these enzymes.

Stability of Streptolysin-Inducing Activity of Ribonucleic Acid.—After adjusting the pH of a series of aliquots of a 5 per cent solution of ribonuclease-treated yeast sodium nucleate, and heating either in a boiling water bath or in the autoclave, the pH was adjusted to neutrality, and the solutions tested for streptolysin-forming activity. All solutions were sterilized prior to testing by immersing them in a boiling water bath for 10 minutes at neutrality. The residual activity of these solutions is shown in Table IV. It can be seen that the stability is greatest at or near neutrality, and that heating at a pH as low as 4.1 or as high as 8.5 caused a considerable amount of inactivation.

Preparation and Properties of Active Fraction (AF) of Yeast Nucleic Acid.—When the digestion mixture resulting from the action of ribonuclease on yeast nucleic acid was dialyzed against water, the streptolysin-inducing activity

failed to dialyze. Making use of this and other properties, a method was developed for the separation of the active portion of yeast nucleic acid:

100 gm. of commercial yeast nucleic acid (Schwarz) is dissolved in 750 cc. water by slowly adding 20 per cent sodium hydroxide, keeping the solution yellow to phenol red. The pH is then adjusted to 7.5, and the volume to 1 liter. The solution is now deproteinized by repeated shaking with chloroform and amyl alcohol according to the procedure of Sevag (6). To each 100 cc. of sodium nucleate solution, 10 mg. crystalline ribonuclease is added. After allowing digestion to proceed at room temperature for 18 hours, the pH is adjusted to 8.0 with 20 per cent sodium hydroxide.

To each 100 cc. of digestion mixture, 10 gm. sodium acetate is added with stirring. Sufficient ethanol is added, with stirring, to bring the concentration to 29 per cent. After 15 minutes, the mixture is centrifuged, and the supernate decanted. Additional ethanol is stirred into the supernate to a concentration of 40 per cent. After centrifugation and removal of the precipitate, the supernate is brought to an ethanol concentration of 50 per cent, re-centrifuged, adjusted to 66 per cent ethanol, and again centrifuged. Each of the four precipitates is dissolved in 50 cc. water, and the solutions tested for streptolysin-forming activity. Most of the activity is present in the fraction precipitating between 29 and 40 per cent ethanol. This fraction is dialyzed in cellophane sacs against running tap water for 18 hours, during which time an increase in volume of approximately tenfold occurs. If a precipitate forms during or after dialysis, it is removed by filtration and discarded. The active material can be preserved in solution in the refrigerator, or, if desired, it can be precipitated with 2 to 3 volumes of ethanol, and dried with ether or acetone. The average yield was 4 to 8 gm. Approximately 0.05 mg. of the product is required to cause the formation of 1000 hemolytic units. The activity per unit weight is about 100 times as great as that of the untreated nucleic acid.

Several lots of yeast nucleic acid were fractionated in the manner described, but with some modifications in the order of the individual steps. Although the active fractions so obtained were not quantitatively identical in regard to content of nitrogen, phosphorus, and activity, certain differences between the active fraction and the parent nucleic acid were consistently observed. These differences are as follows: (a) The nitrogen:phosphorus ratio increased from 1.65 to 1.90–2.30. (b) The purine ribose increased from 23.5 per cent to 26–29.6 per cent. (c) The ultraviolet absorption curve differed significantly from that of ribonucleic acid (Fig. 2). Particularly notable are the shift in absorption maximum from 260 $m\mu$ to 256 $m\mu$, the shift in absorption minimum from 231 $m\mu$ to 226 $m\mu$, and the marked increase in maximum absorption per unit weight of phosphorus. It is evident that AF is a polynucleotide whose constitution differs from that of classical yeast nucleic acid.

Effect of Intestinal Phosphatase on Active Fraction of Yeast Nucleic Acid.—Yeast nucleic acid has been found (18, 19) to be completely dephosphorylated by phosphatase preparations from calf intestinal mucosa. For this reason, the effect of this enzyme on the streptolysin-forming activity of the active fraction of yeast nucleic acid was studied.

Under conditions that were essentially the same as those employed by Zittle (19) in studying yeast nucleic acid, 100 mg. of active fraction of nucleic acid

was incubated at 25°C. with 33 mg. phosphatase in 63 cc. 0.025 M sodium bicarbonate. At the end of 7 hours, it was found that 90 to 95 per cent of the total phosphorus had been converted to inorganic phosphorus. Assay of the digestion mixture for streptolysin-inducing activity showed that 2 to 3 times as much digestion mixture was required to stimulate the same amount of hemolysin formation as was induced by an undigested control solution con-

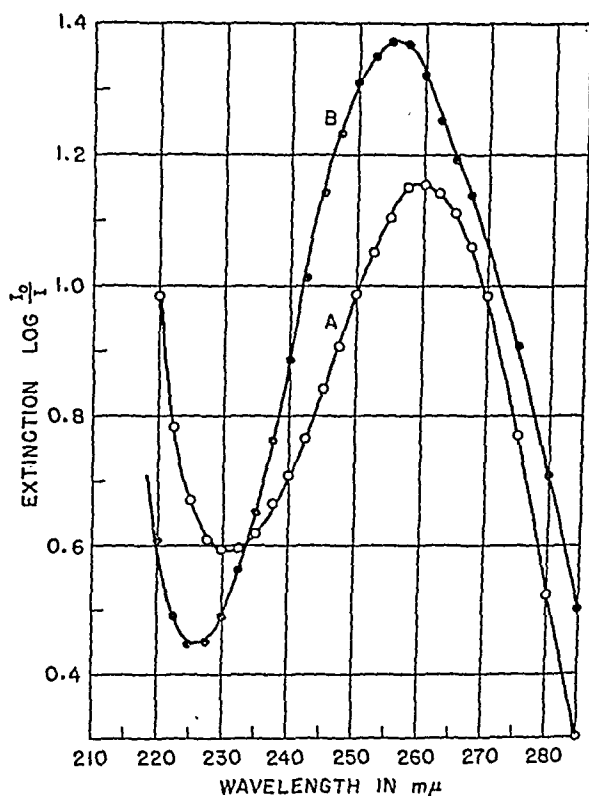


FIG. 2. Ultraviolet absorption spectra of yeast nucleic acid (A) and streptolysin-forming fraction (AF) of yeast nucleic acid (B). Both solutions diluted to contain 3.5 γ phosphorus per cc. Absorption cells, 10 mm.

taining active fraction plus heat-inactivated phosphatase. It is evident that dephosphorylation resulted in a partial loss of activity.

Does Ribonucleic Acid, Regardless of Source, Possess a Ribonuclease-Resistant Fraction?—It has been repeatedly observed (16, 20–22) that yeast nucleic acid is incompletely digested by pancreatic ribonuclease, and that after treatment with the enzyme, there remains a ribonuclease-resistant, non-diffusible portion representing, according to Loring, Carpenter, and Roll (22), about 50 per cent of the original nucleic acid. The present studies show that the streptolysin-inducing activity is associated with the ribonuclease-resistant fraction. Since

the activity of ribonucleic acid was found to depend upon the source (Table II), those preparations from yeast, wheat germ, and streptococci being active, while that of tobacco mosaic virus being inactive, it was of interest to determine whether all of these preparations possessed ribonuclease-resistant fractions.

To approximately 20 mg. yeast sodium nucleate in 2 cc. 0.05 M borate buffer of pH 7.0, was added 1.6 mg. crystalline ribonuclease. The mixture, contained in a cellophane sac, was dialyzed against 1 liter of 0.05 M borate buffer at 22°C., with constant agitation. After 20 hours, the degree of hydrolysis was estimated from the quantity of purine and pyrimidine (absorption at 260 m μ) found in the residue and dialysate, and from analysis of the residue for total phosphorus. Preparations of nucleic acid derived from wheat germ, streptococcus, strain C203S, and tobacco mosaic virus, were treated with ribonuclease under conditions identical with those employed with yeast nucleic acid. In addition, the nucleate solutions were analyzed for desoxyribonucleic acid according to the method of Stumpf (23).

TABLE V

Diffusibility of Products of Ribonuclease-Treated Ribonucleic Acid Derived from Streptococci, Yeast, Wheat Germ, and Tobacco Mosaic Virus

Source of ribonucleic acid	Desoxy- ribonucleic acid content	(A) Proportion of 260 m μ absorbing sub- stance which appeared in dialysate*	(B) Proportion of total P which appeared in dialysate†	A, corrected for content of desoxy- ribonucleic acid‡	B, corrected for content of desoxy- ribonucleic acid‡
	per cent	per cent	per cent	per cent	per cent
Streptococcus C203S	8.5	69	62	75	68
Yeast	0.0	62	70	62	70
Wheat germ	11.5	53	59	60	67
Tobacco mosaic virus	—	62	—	62	—

* Determined in Beckman quartz spectrophotometer.

† Computed by subtracting total P inside of sac after dialysis, from total P before dialysis.

‡ Calculation based on assumption that no desoxyribonucleic acid dialyzed.

|| Not determined.

The results presented in Table V show that enzymatic treatment of the four ribonucleic acid preparations converted to diffusible form 75, 62, 60, and 62 per cent, respectively, of the 260 m μ -absorbing substances, and 68, 70, and 67 per cent of the phosphorus of the first three preparations. It therefore appears that preparations of nucleic acid derived from streptococci, yeast, wheat germ, and tobacco mosaic virus, resemble each other in possessing a ribonuclease-resistant fraction, and one which is present in about equal amount in all of them. It is evident, also, that the presence of a ribonuclease-resistant fraction does not mean that a particular nucleic acid will stimulate the production of hemolysin, since ribonucleic acid of tobacco mosaic virus, although possessing a ribonuclease-resistant fraction, failed to cause hemolysin formation.

II. Carbohydrates

The medium used in the experiments described in the preceding sections was peptone-infusion broth. As has already been stated, it was desired to produce the streptolysin under conditions which are better defined than those provided by this medium. For this purpose, a medium whose chemical composition is essentially defined (4) was substituted for peptone-infusion broth. In the defined medium, however, the streptococci produced relatively little streptolysin in the presence of an excess of AF, even though bacterial growth was comparable to that in peptone-infusion broth. Occasionally, cultures grown in defined medium containing AF produced 500 or more units of streptolysin

TABLE VI
Streptolysin-Promoting Effect of Peptone-Infusion Broth

Additions to defined culture medium	Hemolytic units per cc. of culture
None.....	<100
1 cc. YNA digest.....	<100
1 cc. YNA digest + 1 cc. peptone-infusion broth.....	1750
3 cc. peptone infusion broth.....	<100
1 cc. YNA digest + 1 cc. 1 per cent peptone.....	1000
1 cc. YNA digest + 1 cc. meat infusion.....	2000
1 cc. YNA digest + 0.5 cc. dialyzable fraction of meat infusion.....	1000
1 cc. YNA digest + 1 cc. non-dialyzable fraction of meat infusion.....	1000

Each tube contained 9 cc. defined medium (4), 0.05 per cent glucose, and 0.01 cc. inoculum.

YNA digest: 1 per cent solution of yeast nucleic acid containing 0.01 per cent ribonuclease.

Peptone-infusion broth prepared as described in Methods.

All solutions were sterilized at 15 pounds steam pressure for 15 minutes, excepting dialysis fractions which were sterilized at 100°C. for 10 minutes.

per cc., but the formation of this much streptolysin was unusual and not reproducible. When, however, a sufficient amount of peptone-infusion broth was added to the defined medium, the effect of AF was consistently demonstrable. It also was clear that, in the absence of AF, no amount of peptone-infusion broth was effective in inducing a significant degree of streptolysin formation. These observations suggested that peptone-infusion broth contains a factor, or factors, other than ribonucleic acid, necessary for the formation of streptolysin. When peptone and meat infusion were tested separately, it was found that either supplied the missing factor(s). The factor(s) in both materials withstood autoclaving at or near neutrality. Approximately 70 per cent of the activity of meat infusion was found to be dialyzable and was recoverable from the dialysate. Experimental data illustrating most of these results are summarized in Table VI.

Additional experiments showed that: (1) heating meat infusion at 100°C. for 60 minutes at pH 12 destroyed approximately three-fourths of the streptolysin-promoting activity; (2) heating the infusion at the same temperature for the same length of time at pH 1 resulted in little or no change in activity; (3) incineration of meat infusion destroyed completely the streptolysin-promoting activity.

Streptolysin-Promoting Effect of Maltose.—In order to find out whether they could replace meat infusion, a number of substances believed to be present in meat infusion, and having properties similar to those just described, were tested for streptolysin-promoting activity. It was found that very low concentrations of maltose consistently induced the formation of streptolysin in defined medium containing AF. The effect of maltose on streptolysin production is shown in Table VII. In studying streptolysin formation as a function

TABLE VII
Streptolysin-Promoting Effect of Maltose

Final concentration of maltose in medium	AF	Hemolytic units per cc. of culture
	cc.	
M/4000	0.1	2200
M/8000	0.1	2200
M/32,000	0.1	900
M/64,000	0.1	150
None	0.1	<100
M/4000	None	<100

Medium as described in reference 4 but containing 0.05 per cent glucose and 0.2 per cent potassium bicarbonate.

of maltose concentration, the amounts of streptolysin formed in response to the very lowest maltose concentrations were found not to be quantitatively reproducible. However, as little as M/64,000 maltose usually had a detectable effect, provided the initial concentration of glucose was M/360 and an excess of AF was present. The maximum yield of streptolysin, 1500 to 3000 units per cc. of culture, was obtained when the initial maltose concentration was approximately M/20,000. Higher concentrations sometimes depressed the yield. It was observed, also, that maltose was not effective in promoting streptolysin formation when the medium was deficient in AF (Table VII).

Effect of α -Glucosidase on Streptolysin-Promoting Action of Maltose.—Unless the effect of maltose is due to an impurity, it follows that enzymatic hydrolysis of maltose will destroy its streptolysin-promoting action.

To 20 cc. M/80 maltose in M/60 phosphate buffer, pH 7, was added 10 cc. water containing 10 mg. of a preparation of α -glucosidase. After setting the mixture at 30°C., 1.5 cc. aliquots

were removed at intervals, placed in boiling water for 5 minutes, and chilled. After bringing the volume of each aliquot to 5 cc., 0.1 cc. was tested for streptolysin-inducing activity, employing defined medium deficient in maltose but containing AF. In addition, each aliquot was tested for reducing sugar according to the method of Nelson (24), and from the reducing values, the extent of maltose hydrolysis was computed. Curves showing streptolysin-inducing action as a function of time, and maltose hydrolysis as a function of time are given in Fig. 3.

It can be seen that in 10 minutes, when approximately 45 per cent of the maltose had disappeared, there was an appreciable reduction in streptolysin-inducing activity, while at 60 minutes, when approximately 75 per cent of the maltose had disappeared, there no longer was stimulation of streptolysin formation. It is clear that hydrolysis of maltose was accompanied by disappearance of streptolysin-inducing capacity.

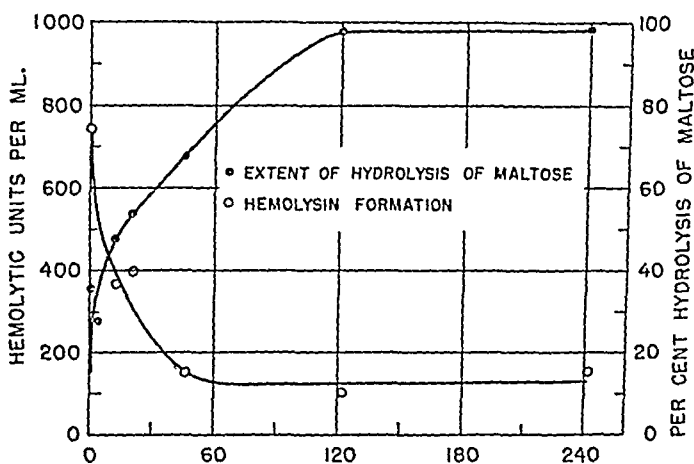


FIG. 3. Effect of α -glucosidase on streptolysin-promoting capacity of maltose. Abscissa: Time of hydrolysis of maltose in minutes at 37°C., pH 7.0. Zero time corresponds to $M/40,000$ maltose.

Specificity of Maltose.—In order to investigate the specificity of maltose in causing streptolysin formation, a considerable number of carbohydrates were tested.

After sterilizing by immersion in boiling water for 10 minutes, solutions of carbohydrates were added singly to defined medium containing AF and $M/360$ glucose. Most of the sugars and simple sugar derivatives were tested in final concentrations of $M/4000$ and $M/40,000$, while most of the polysaccharides were tested in final concentrations of 1 mg. and 0.1 mg. per cc. Additional concentrations were tested when the results indicated this to be desirable. Maltose, in a concentration of $M/30,000$, produced approximately 1000 units of streptolysin per cc., and it was arbitrarily assigned a "streptolysin-producing value" of 100. The relative activities of other carbohydrates were computed from the concentrations required for the formation of 1000 units of streptolysin. When the molecular weights were known, the relative activities were reckoned on a molar basis; otherwise on a weight basis. The streptolysin-inducing capacity of the substances tested is shown in Table VIII.

TABLE VIII

Streptolysin-Inducing Capacity of Carbohydrates and Related Substances

	Activity relative to maltose
Monosaccharides	
<i>d</i> -Glucose	<5
<i>d</i> -Mannose	10
<i>l</i> -Rhamnose	<5
<i>d</i> -Fructose	<5
<i>d</i> -Galactose	<5
<i>d</i> -Arabinose	<10
<i>d</i> -Ribose	<2
<i>d</i> -Xylose	<5
Disaccharides	
Maltose (4- <i>d</i> -glucopyranosyl- α - <i>d</i> -glucopyranoside)	100
Lactose (4- <i>d</i> -glucopyranosyl- β - <i>d</i> -galactopyranoside)	<5
Sucrose (1- α - <i>d</i> -glucopyranosyl- β - <i>d</i> -fructofuranoside)	<5
Trehalose (1- α - <i>d</i> -glucopyranosyl- α - <i>d</i> -glucopyranoside)	20
Cellobiose (4- <i>d</i> -glucopyranosyl- β - <i>d</i> -glucopyranoside)	<5
Melibiose (6- <i>d</i> -glucopyranosyl- α - <i>d</i> -galactopyranoside)	<5
Tri- and polysaccharides	
Raffinose	10
Glycogen (beef liver)	ca. 2
Glycogen (oyster)	ca. 2
Starch	ca. 2
Dextrin	10
Inulin	<10
Potassium hyaluronate (umbilical cord)	2
Potassium hyaluronate (vitreous humor)	<1.5
Chondroitin sulfate	<1.5
Hexahydric alcohols	
Inositol	<5
Dulcitol	<10
Mannitol	<5
Sorbitol	<5
Miscellaneous hexose derivatives	
Sodium thioglucose	<5
Glucosamine	30-50
<i>N</i> -Acetylglucosamine	<10
Glucose-1-phosphate	<5
Fructose-6-phosphate	<5
Fructose-1,6-diphosphate	<5
α -Methylglucoside	<5
Salicin	<5
Calcium maltobionate	<5

We are indebted to Dr. W. F. Goebel for supplying us with mammalian glycogen, to Dr. M. Levy for oyster glycogen, to Dr. M. P. Schubert for sodium thioglucose, *N*-acetylglucosamine, potassium hyaluronate, and chondroitin sulfate, and to Dr. F. H. Stodola for calcium maltobionate.

It is evident that none of the substances tested was as active as maltose. Approximately 10 times as much dextrin, and about 50 times as much glycogen, were required to produce the same effect as maltose. Of the sugars having configurations most closely resembling that of maltose, only trehalose was found to be active, and its activity was about one-fifth that of maltose. None of the pentoses was found to be active, and of the hexoses tested, only *d*-mannose possessed significant activity. It was about one-tenth as active as maltose. Other than maltose and trehalose, none of the α -glucosides, hexahydric alcohols, or phosphorylated hexoses tested was found to be active. In contrast to these findings, glucosamine proved to be highly active. In concentrations of $M/10,000$ to $M/15,000$, it produced as great an effect as $M/30,000$ maltose. *N*-Acetylglucosamine was found to have little or no activity.

DISCUSSION

Particular attention has been paid to the specificity of ribonucleic acid in inducing hemolysin formation, and the results show that no other substance tested, including desoxyribonucleic acid as well as acid and alkaline hydrolysis products of ribonucleic acid, produces the same effect as ribonucleic acid. It should be noted, however, that not all preparations of ribonucleic acid are active. The activity apparently depends upon the source. Preparations of ribonucleic acid derived from yeast, wheat germ, streptococci, and mammalian liver, were found to be active, while nucleic acid derived from tobacco mosaic virus was found to be inactive. It appears significant that after treatment with ribonuclease, the active preparations possessed, on a weight basis, approximately equal activity.

Attention may be called to the observation that ribonucleic acid prepared from the test strain of streptococcus, C203S, and subsequently treated with ribonuclease, was found to be approximately as active as ribonuclease-treated nucleic acid prepared from yeast. It is evident, therefore, that the cocci contain ribonucleic acid having potential streptolysin-forming activity, and it is evident, also, that this nucleic acid must have been synthesized by the streptococci because the cocci had been cultivated in a medium free of nucleic acid.

Since the quantity of streptococcal growth attained in a broth culture is less than 0.5 mg., dry weight, per cc., and since approximately 15 per cent (25) of the dry weight of streptococci is ribonucleic acid, it can be calculated that the concentration of streptococcal ribonucleic acid in fully grown broth cultures is not greater than 0.075 mg. per cc. Reference to Fig. 1 shows that this concentration of ribonucleic acid is only one-thirtieth of that necessary to induce the formation of 1000 hemolytic units. It is therefore not surprising to find that plain broth cultures of streptococci usually contain less than 100 hemolytic

units per cc., even though the cocci are able to synthesize nucleic acid which is potentially capable of inducing streptolysin formation.

The finding that nucleotides and their hydrolysis products fail to replace ribonucleic acid in forming streptolysin confirms statements to the same effect published by Itô (26). However, our finding that desoxyribonucleic acid is inactive, contrasts with observations made by Itô, in whose experience desoxyribonucleic acid prepared from cow spleen could replace yeast ribonucleic acid.

Fractionation of yeast nucleic acid shows that the activity is present in the ribonuclease-resistant portion, and with this knowledge, it has been possible to prepare a polynucleotide (AF) possessing activity many times greater than that of the starting material. The increased nitrogen:phosphorus ratio of AF, and the increased content of purine ribose, are in agreement with recent observations of Loring, Carpenter, and Roll (22) and with those of Schmidt, Cubiles, Swartz, and Thannhauser (27) on the nature of action of ribonuclease on yeast nucleic acid.

There remains the question of whether the activity studied in the present investigation is actually due to a fraction of ribonucleic acid or to a contaminating substance which tends to be coprecipitated with ribonucleic acid. In regard to yeast nucleic acid, it seems likely from the data reported in the foregoing section, that the streptolysin-inducing effect is caused by an integral component of yeast ribonucleic acid itself. In any event, it is clear that the active substance is either (1) a portion of the ribonuclease-resistant polynucleotide, or (2) a substance which is closely associated with that component. We are inclined to favor the former view, but it must be admitted that the evidence does not conclusively eliminate the second possibility.

The biological importance of desoxyribonucleic acids is clearly indicated by Avery, MacLeod, and McCarty's demonstration (28) of the capacity of desoxyribonucleic acid to direct specifically the synthesis of pneumococcal capsular polysaccharide. Although ribonucleic acid also is commonly believed to play an important rôle in cellular processes, there are but few instances in which its functions have been defined. For this reason, Okamoto's discovery of a specific biological effect of yeast nucleic acid seems to merit greater attention than has been accorded it hitherto. The results of the present study confirm and extend Okamoto's observation of the capacity of yeast nucleic acid to stimulate the formation of a potent streptolysin, and, in so doing, provide a technique which may prove useful in the study of nucleic acid structure and metabolism.

In regard to the specificity of maltose in promoting streptolysin formation, the results summarized in Table VIII show that of the disaccharides tested, only maltose and trehalose were active. It may be noted that both are α -glucosides. Although α -galactoside (melibiose) as well as disaccharides having the β -configuration were not active, the number of α -glucosides tested is so

small that no conclusion can be drawn concerning the activity of α -glucosides in general. It is of interest that cellobiose, a β -glucoside, but otherwise closely resembling maltose in structure, was not active. The streptolysin-promoting activity of trehalose suggests that a free aldehyde group is not essential for activity. The results are somewhat complicated by the finding that mannose possessed some activity, and still more by finding that glucosamine was almost as active as maltose.

There are on record several observations which show that certain carbohydrates favor the production of toxins by bacteria. In the production of diphtheria toxin, maltose has long been used in preference to glucose as the chief energy-supplying compound (29). In studying the production of α -toxin of *Clostridium welchii*, Logan, Tytell, Danielson, and Griner (30) have shown that the best yields of toxin are obtained when the medium contains dextrin as the sole or principal carbohydrate. This observation has been confirmed by Adams, Hendee, and Pappenheimer (31). In both investigations, it was observed that maltose was less effective than dextrin, although more effective than glucose, in promoting toxin production. It is not known whether a common mechanism underlies these effects on the production of toxin by different bacteria.

On the basis of the information presented, it is scarcely permissible to speculate on the rôle of AF and of carbohydrates in promoting streptolysin formation. There is one point, however, which is worthy of comment. The results show that very small amounts of either maltose or glucosamine can be detected in the presence of relatively large amounts of glucose. In the case of maltose, 6 γ per cc. produces a detectable effect in a culture containing, initially, 80 times as much glucose. Although the streptococci can utilize either maltose or glucosamine as a source of energy, it may be questioned whether they do so in the presence of a relatively high concentration of glucose. The possibility that maltose and glucosamine are not oxidized, but are used instead, as units from which larger molecules are synthesized, should be considered.

As a consequence of the present study, it is possible to produce the nucleic acid-induced streptolysin under comparatively well defined conditions. Using a peptone-free medium, supplemented with appropriate amounts of AF, maltose, and glucose, streptolysin has been obtained in fair yield in cultures of 5 liters in volume. The hemolytic titers obtained in this medium have been about half as great as those which can be got in peptone-infusion broth containing AF. The conditions required for maximum streptolysin production, therefore, are still not completely defined. The peptone-free medium, however, affords advantages which offset the disadvantage of a reduced yield. For example, by employing this medium, the nucleic acid-induced streptolysin has been prepared in concentrated, partially purified form with relatively little difficulty.

SUMMARY

1. Ribonucleic acid of yeast causes the formation of a potent hemolysin in broth cultures of *Streptococcus pyogenes*.
2. The hemolysin whose formation is induced by yeast ribonucleic acid appears to be identical with streptolysin S.
3. Desoxyribonucleic acid, products of acid or alkaline hydrolysis of ribonucleic acid, or many other substances tested, fail to produce a similar effect.
4. Digestion by ribonuclease increases markedly the streptolysin-inducing activity of certain preparations of ribonucleic acid.
5. A fraction (AF) of yeast nucleic acid has been isolated which possesses approximately 100 times the streptolysin-inducing capacity of the starting material. Some of the properties which distinguish AF, a polynucleotide, from ordinary yeast nucleic acid are described. AF is associated with the ribonuclease-resistant fraction of yeast nucleic acid.
6. Ribonucleic acid prepared from streptococci, wheat germ, and mammalian liver, and subsequently treated with ribonuclease, is about as active in causing streptolysin formation as ribonuclease-treated yeast nucleic acid.
7. Ribonucleic acid of tobacco mosaic virus, tested under comparable conditions, was found to be inactive.
8. Ribonucleic acid prepared from streptococci, wheat germ, and tobacco mosaic virus resembles yeast nucleic acid in possessing a ribonuclease-resistant fraction.
9. In addition to AF, a factor (or factors), present in meat infusion and in peptone, was found to be required for the formation of streptolysin.
10. The factor can be partially replaced by any one of several carbohydrates, the most active being maltose, glucosamine, and trehalose, in that order.
11. When appropriate concentrations of AF, maltose, and glucose are used, the nucleic acid-induced streptolysin can be produced in a medium whose chemical composition is essentially defined.

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ESTERASES OF TESTIS AND OTHER TISSUES*

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This paper is concerned with some physiologic relationships of non-specific esterases of tissues of the rat, dog, and man which were investigated by a colorimetric technique. Two patterns of hydrolysis were revealed that were characteristic of several tissues. It was found also that the esterase of rat testis is produced in the interstitial cells, that its content is under control of the hypophysis, and that its concentration is related to the production of androgen by the testis.

The colorimetric method employed chromogenic substrates, colorless compounds that liberate color on hydrolysis. The color value is read directly, the depth of color produced by enzymatic hydrolysis being directly related to activity of the enzyme. The chromogenic substrate techniques of enzymatic investigation combine accuracy, delicacy, and simplicity. All the chromogenic substrates, beginning with the first use of this technique by Ohmori (1), have been compounds of phenolic acid-base indicators esterified with phosphoric (1, 2), glucuronic (3), sulfuric (4), or fatty acids (5). In the present studies the substrates were the acyl esters of *p*-nitrophenol previously synthesized in this laboratory.

Three principal esterase activities in animal tissues can be differentiated according to their distribution, their efficiency against various substrates, and the effect of inhibitors (5, 24) upon them. An enzyme cannot be characterized with certainty until it has been isolated in a pure state and this has not been achieved with any of the esterases. However, from indirect evidence esterolysis seems to be brought about by three separate types of enzymes; in this paper the effects will be designated according to conventional usage as cholinesterase, non-specific esterase, and lipase. No doubt there is some overlapping of their activities and, as will be brought out, the chief differentiating characteristics are quantitative rather than qualitative.

The choline esters are sufficiently soluble in water to serve as satisfactory substrates *in vitro*. Cholinesterase has an entirely different distribution than the other esterases. Marnay and Nachmansohn (6) found that the cerebral cortex and uterus of guinea pig hydrolyzed acetylcholine more effectively than extracts of kidney or liver did. In swine (7) acetylcholine is hydrolyzed more rapidly by certain structures which, in descending order of magnitude, are the parotid, lachrymal and sublingual glands, Fallopian tube, jejunal and gastric mucosa, and the medulla oblongata. Mendel,

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Mundell, and Rudney (8) further differentiate two types of cholinesterase activity by their effect on various choline esters—true and pseudocholinesterase. According to their findings, both enzymes hydrolyze acetylcholine; acetyl- β -methylcholine is hydrolyzed by true but not by pseudocholinesterase while benzoylcholine is hydrolyzed by pseudo- but not by true cholinesterase. Sawyer and Everett (9) found that the tissues of the rat with the greatest hydrolytic activity against benzoylcholine were the salivary and Harderian glands, brown fat, ovary, uterus, and liver; acetyl- β -methylcholine was hydrolyzed best by the brain stem, red bone marrow, spleen, thymus, lymph node, adrenal cortex, skeletal muscle, and peripheral nerve. Other choline esters have also been used as substrates. Stedman, Stedman, and White (10) observed that butyrylcholine is hydrolyzed by serum of various animals about twice as rapidly as acetylcholine. Nachmansohn and Rothenberg (11) found that the brain of rodents, ox, and cat likewise showed hydrolytic patterns; no choline substrate was hydrolyzed by these tissues more rapidly than acetylcholine; propionylcholine was hydrolyzed equally well or less than acetylcholine while butyrylcholine was split much less. Extracts of the electric organ of the eel (5) were found to be 600,000 times more effective in splitting acetylcholine compared to the hydrolysis of *p*-nitrophenyl propionate. This is evidence that the hydrolysis of these acyl esters of *p*-nitrophenol is performed by enzymes other than cholinesterase.

The distribution of esterases active on non-choline esters has not been systematically investigated. Moreover, earlier methods have presented technical difficulties since the fats and esters of fatty acids have only slight solubility in water. Except for the lipase method of Archibald (12) and our chromogenic technique, all previous quantitative studies have been done on suspensions or emulsions in which, to a large extent, the enzyme and the substrate have been in different phases. In comparative studies (13) employing different substrates the possibility has existed that the "results merely portray the degree of emulsification attained." In the earlier literature the terms lipase and esterase often have been used interchangeably although, as will be brought out, the enzymes are apparently separate entities.

In the activity of non-specific esterase and lipase the character of the fatty acid has far greater influence on the reaction than the hydroxyl-bearing group. Kastle (14) observed that the alkyl groups methyl, ethyl, iso-butyl, allyl, and benzyl exert nearly the same influence on the hydrolysis of esters by aqueous extracts of liver. Balls and Matlack (13) tested the effect of pancreatic extracts on fatty acids of various alcohols. The configuration of the alcohol was without effect on the enzyme except in so far as the hydroxyl-bearing carbon was concerned; esters of primary alcohols were split effectively but secondary and tertiary alcoholic esters were attacked slowly.

Most of the work done on non-specific esterase of cells has concerned liver and pancreas, tissues which differ widely from the esterase standpoint. Kastle and Loevenhart (15) observed that ethyl butyrate is hydrolyzed by extracts of liver, pancreas, kidney, and submaxillary gland; pancreatic extracts hydrolyzed in decreasing order ethyl esters of butyric, propionic, and acetic acids. Kastle (14) reported a pattern similar to that of pancreas for liver extracts, a finding in conflict with our observations using *p*-nitrophenyl esters. Loevenhart (16) found that liver extracts were several times as active as pancreas on esters of the lower fatty acids while the reverse was true with higher fatty acids. Balls and Matlack (13) found that pancreatic extracts hydro-

lyzed not only glycerides of fatty acids but esters of monatomic alcohols as well and at approximately the same rate as the saturated fats. Pancreatic extracts hydrolyzed many esters of stearic acid but extracts of horse liver, while rich in esterase (ethyl butyrate-splitting), had small or no activity against stearate esters. Both liver and pancreas split the lower esters but only the pancreas hydrolyzed esters of long chain acids efficiently. That esters of long chain fatty acids can be split by tissues other than pancreas was demonstrated by Gomori (17, 18) in his histochemical demonstration of lipase. Using polyglycol esters of palmitic and stearic acids he obtained intensely positive reactions in the liver of all species and the interstitial cells of the rat testis as well as in the zymogen granules of pancreatic cells. Each of these tissues, therefore, had some capacity to hydrolyze long chain esters.

Falk, Noyes, and Sugiura (19) employed miscellaneous esters of acetic, butyric, and benzoic acids in testing esterase activity of rat tissues and a transplantable carcinoma. They found that the esterase content of the tumor was small while the greatest activity was in kidney and liver, with testis following closely and then spleen and lung; the activity curves show characteristic "pictures" for the ester-hydrolyzing actions of the tissues.

Methods

All the rats utilized were exsanguinated by cardiac puncture and samples of their tissues (about 100 to 150 mg.) were then weighed on a torsion balance and homogenized in an all-glass grinder in 5 ml. of ice-cold water. Tissues obtained from two dogs killed by electrocution were treated similarly. Certain fresh human tissues were obtained directly from an operating room. The homogenates were centrifuged and aliquots of the supernatant fluid were diluted with water in volumetric flasks.

The esterase technique was that described previously (5) except that it was carried out at a temperature of 30°; each analysis was done in triplicate. One unit of esterase activity is defined as that amount of enzyme liberating one micromole of *p*-nitrophenol in 20 minutes at 30° and pH 7 in phosphate buffer when the substrate is at a concentration of 0.666 micromole per 10 ml., provided that not more than 40 per cent of the substrate is hydrolyzed; the units are expressed per 1 gm. of tissue or 1 ml. of fluid.

The following esters of *p*-nitrophenol were used: acetate, propionate (PNPP), *n*-butyrate (PNPB), *n*-valerate. In all cases the same molarity of substrate was employed. In preparing aqueous solutions of *p*-nitrophenyl-*n*-valerate precipitation frequently occurred; the precipitation was removed by filtering several times through a No. 40 Whatman filter paper. Under ether anesthesia hypophysectomy was done in 42 rats using the parapharyngeal technique of Smith (20). Equine gonadotrophin¹ was freshly dissolved in saline before intramuscular injection and was administered after some days to 15 hypophysectomized rats.

RESULTS

Survey of Tissues Esterase.—In the rat the greatest concentration (Table I) of non-specific esterase (substrate: *p*-nitrophenyl propionate) was found in five tissues: liver, lung, pancreas, adult testis, and renal cortex. In the dog these same tissues were rich in esterase; in addition, the mucosa of the trachea

¹ The authors are indebted to the Upjohn Company, Kalamazoo, for the generous gift of this material, distributed by them under the trade name gonadogen.

and urinary bladder were exceptionally active and gastric mucosa had moderate activity. The lens of the dog had appreciable esterase activity. While all tissues examined had some esterase activity, thymus, spleen, bone marrow, and heart muscle were relatively inactive and skeletal muscle had very slight activity.

Esterase Patterns of Tissues.—The five tissues of the rat found to have the greatest esterase activity were tested simultaneously against *p*-nitrophenyl

TABLE I
Esterase Content of Tissues of Rat and Dog

Substrate: *p*-nitrophenyl propionate.

	Rat	Dog
	Units per gm. or cc.	
Liver.....	823 - 3460	1218 - 2680
Lung.....	332 - 449	156 - 298
Tracheal mucosa.....	74 - 117	587 - 685
Pancreas.....	241 - 584	194 - 526
Testis.....	224 - 380	18 - 29
Kidney, cortex.....	205 - 368	181 - 332
Kidney, medulla.....	28 - 76	3 - 14
Bladder mucosa.....	17 - 48	507 - 2640
Pituitary.....	63.8	123 - 174
Cerebrum.....	20 - 37	8 - 25
Cerebellum.....	13 - 20	4 - 20
Suprarenal.....	25 - 75	9 - 16
Thyroid.....	17 - 50	46 - 239
Gastric mucosa.....	19 - 30	228 - 252
Spleen.....	14 - 26	9 - 75
Prostate, ventral.....	33 - 138	57 - 230
Thymus.....	18 - 23	—
Heart.....	21 - 25	14 - 18
Skeletal muscle.....	3 - 6	10 - 34
Lens.....	6	26 - 46
Bone marrow.....	8 - 14	—
Blood serum.....	7 - 9	4.7 - 7

esters of straight chain fatty acids containing 2 to 5 carbon atoms in the chain. Definite hydrolytic patterns emerged which were of two types. In one pattern propionic acid esters (C_3) were split preferentially by liver, lung, testis, and renal cortex; acetate esters (C_2) lagged slightly, while butyrate (C_4) and valerate (C_5) esters were attacked (Table II) with decreasing effectiveness. Profiles of blood serum of the dog, rabbit, and man (5) were similar to those of these tissues.

The pancreas of dog and rat was quite different in its effects. The splitting

of acetate esters (C_2) was feeble (Fig. 1) but there was a progressive increase of activity with chain length until valerate esters (C_6) were reached.

In the tissues of 3 infant rats 5 days of age patterns of hydrolysis were obtained which were identical with those of the corresponding adult tissues.

Esterases in Postnatal Development.—The non-specific esterases of tissues of the newborn rat were low in amount. In rats at age 4 and 5 postnatal days

TABLE II

Patterns of Hydrolysis of Esters of p-Nitrophenol by Rat Tissues

Values expressed as per cent of the ester split maximally.

Determination No.	p-Nitrophenyl			
	acetate	propionate	n-butyrate	n-valerate
<i>Liver</i>				
1	95	100	62.4	41
2	94	100	85	76
3	88	100	71	25
<i>Lung</i>				
1	74	100	84	48
2	59	100	58	26
3	80	100	62	31
<i>Testis</i>				
1	92	100	87	32
2	83	100	53	23
3	96	100	80	46
<i>Renal Cortex</i>				
1	81	100	39	45
2	91	100	36	40
3	88	100	58	52
<i>Pancreas</i>				
1	3.5	19	22	100
2	6.3	29	38	100
3	3.2	16	52	100

average esterase (p-nitrophenyl propionate) values were found as follows: lung 10 units; renal cortex 80 units; liver 160 units. Compared to these were the average findings in litter mates at 13 days: lung 304 units; renal cortex 206 units; and liver 725 units.

The esterase values of umbilical cord blood of children at birth also were very low; in 12 newborn babes the value ranged from 1.53 to 2.37 units per ml. while the blood of the mothers taken at the same time by venipuncture ranged from 4.12 to 8.15 units.

The esterase of rat testis between age 5 and 27 days (Fig. 2) varied between 5 and 14 units per gm.; between age 27 and 29 days the esterase doubled and

there was a sharp and progressive rise to 35 days, when adult levels were encountered. In albino rats raised in this laboratory, spermatozoa are found in the testis between age 34 and 37 days.

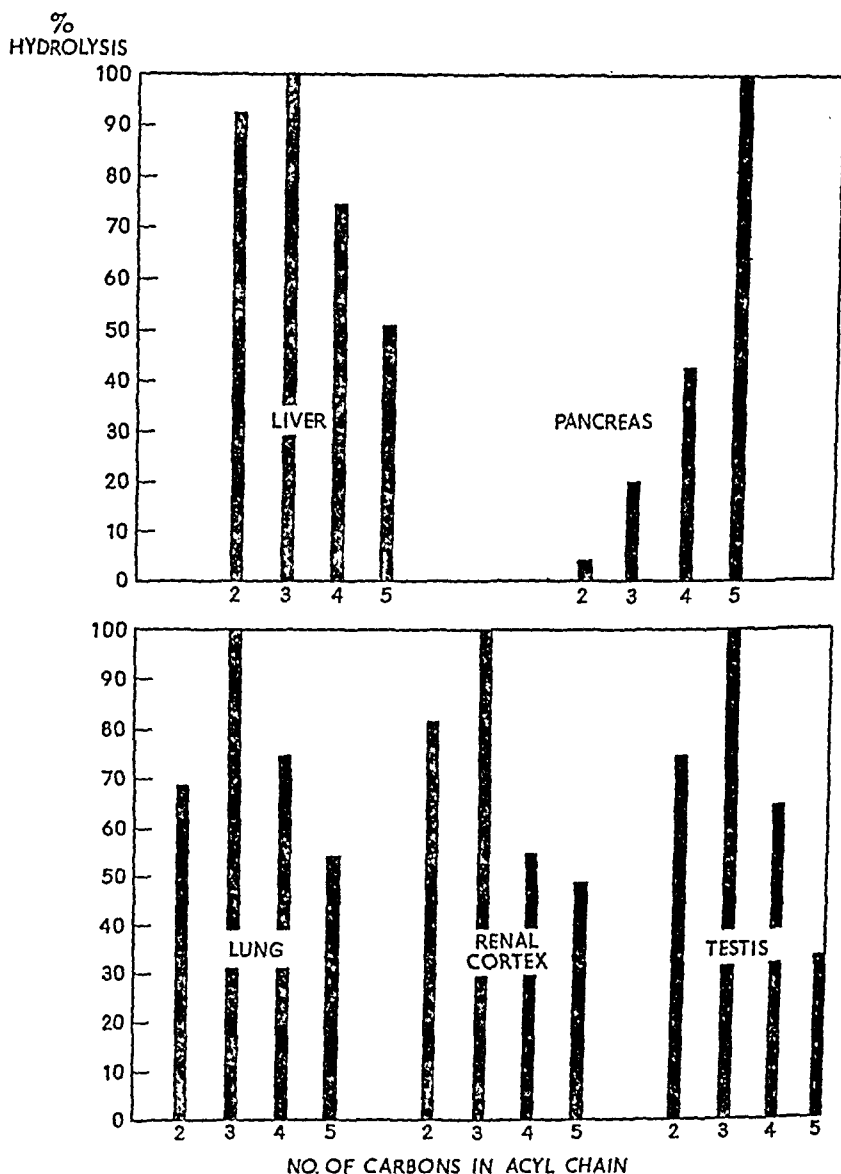


FIG. 1. Patterns of enzymatic hydrolysis of acyl esters of *p*-nitrophenol by tissues. Acetate (C_2), propionate (C_3), *n*-butyrate (C_4), and *n*-valerate (C_5) esters were used as substrates in equimolar concentration and were tested simultaneously. Average values are given and the results are expressed in percentage of that ester hydrolyzed at the fastest rate.

Some preliminary assays of human tissues may be noted for comparison. The esterase of the testes of 6 men between 55 and 70 years of age ranged from

2.06 to 7.61 units. A few isolated normal tissues were studied: renal cortex 44 units; bladder mucosa 29 units; colon mucosa 39 units.

Effect of Cryptorchism on Testicular Esterase.—In 18 rats the left testis was anchored through an abdominal incision by suture to the anterior surface of

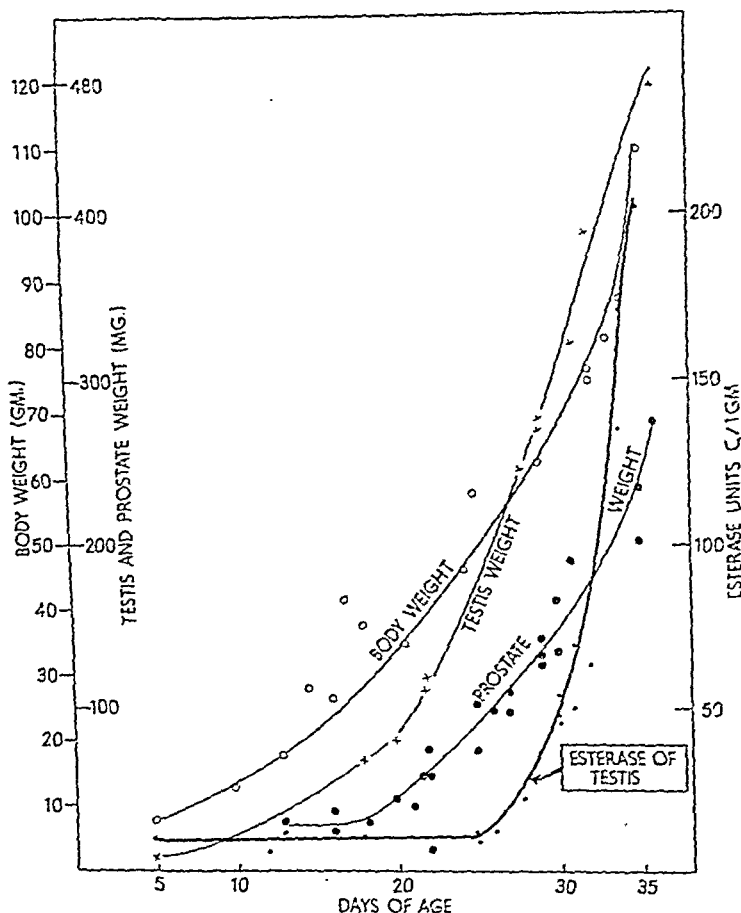


FIG. 2. Growth curves of development of testis, ventral prostate, and body weight of the rat. The concentration of non-specific esterase (substrate *p*-nitrophenyl propionate) is indicated in the curve on the right.

the peritoneal cavity; 6 to 8½ weeks later the cryptorchid testis and its normal mate were recovered for esterase determination. The concentration of esterase was increased in the abdominal testis (Table III) compared to the control which was not operated upon; the total amount in the cryptorchid testis progressively decreased with time from 89 to 50 per cent of the normal.

Testicular Esterases after Hypophysectomy and Gonadotrophin Injection.—Two weeks after hypophysectomy both the content and concentration of the testicular esterase were found to be markedly decreased (Table IV) as compared with litter mate control rats. It is perhaps significant that the low values of early puberty (before 27 days) were not reached, so from an enzymatic standpoint the testis of a hypophysectomized rat differs from the testis of the infant.

TABLE III
Esterase in Normal and Cryptorchid Testes

No.	Post-operative	Normal testis			Cryptorchid testis			Total esterase ratio Cryptorchid Normal
		Weight	Esterase	Total esterase	Weight	Esterase	Total esterase	
	days	gm.	units/gm.	units/gm.	gm.	units/gm.	units/gm.	
1	42	1.444	144	208	0.643	288	185	0.89
2	56	1.367	533	729	0.680	636	432	0.59
3	60	1.836	575	1056	0.521	1021	532	0.50
4	60	1.373	409	562	0.411	753	309	0.55

TABLE IV
Effect of Hypophysectomy and Gonadotrophin on Esterase of Testis

Experiment No.	Length of time after hypophysectomy	Gonadotrophin	Body weight	Testis weight		Esterase		
						Prostate	Testis	Testis
	days	units	gm.	mg.	mg.	units/gm.	units/gm.	units/gm.
1	Unoperated control	None	178	1169	1194	33	258	332
2	Unoperated control	None	220	1210	1230	112	380	413
3	16	None	140	744	821	89	52	40
4	18	None	155	890	912	17	27	53
5	21	30	132	396	408	133	133	138
6	22	40	160	896	904	54	250	255

Equine gonadotrophin, 10 units daily for 3 and 4 days, caused the content and concentration of esterase to increase in the testis (Table IV), but neither the esterase values nor the weight of the testis approached the normal values.

DISCUSSION

The distribution of non-specific esterase was found to be quite different from that of cholinesterase. Cholinesterase in the rat has previously (9) been found to have its greatest concentration in certain small glands, the uterus, and the brain stem, while our observations show that non-specific esterase has its greatest accumulation in liver, lung, testis, renal cortex, and pancreas. These findings are interpreted as additional evidence for the dissimilarity of non-

specific esterase and cholinesterase. It is of interest that the lens, a tissue of sluggish metabolic activity, has an appreciable esterase content.

Two patterns emerge from differences in tissues in their hydrolysis of fatty acid esters of varying chain length. In one pattern, characteristic of lipase, there is increasing hydrolytic activity with increases of the acyl chain from 2 to 5 carbons. This pattern was found in the pancreas of several kinds of animals, namely, the dog, rabbit, and rat, and elsewhere only in rat serum. The serum of the rat is more effective in splitting C_2 and C_3 acid esters than is pancreas, no doubt because it contains appreciable quantities of non-specific esterase, but the predominant effect resembles pancreas more than liver.

In the other hydrolytic pattern the 3-carbon acyl ester (propionate) is split more effectively than the other members of the series. This pattern is exhibited in the rat by extracts of liver, lung, renal cortex, and testis and also by dog, human, and rabbit serum. This pattern we consider to be characteristic of non-specific esterase.

It is possible to predict the pattern of hydrolysis of acyl esters of *p*-nitrophenol by testing any extract against propionate (PNPP) and butyrate (PNPB) esters. If PNPP is split faster than PNPB, acetate is always split slower than propionate and valerate is hydrolyzed more slowly than butyrate. When PNPB is hydrolyzed faster than PNPP, acetate is split less effectively than propionate and valerate esters are split faster than butyrate. Acetate esters of *p*-nitrophenol are always hydrolyzed less effectively by non-specific esterase and lipase than propionate esters.

The patterns in infant tissues do not differ from those of the adult—merely quantitative differences occur. The tissues of the infant rat (4 to 5 days) are very low in non-specific esterase. Concerning enzymes of young animals, it is known that very young fetuses of the goat contain extraordinarily little carbonic anhydrase (21) and this enzyme in the blood of newborn infants (22) is less than half that found in the blood of adults. Esterase in rat tissues other than the testis accumulates rapidly so that at 12 to 18 days normal values are found. The testicular esterase remains at low and fairly regular levels until age 27 days when it increases markedly in content and concentration, reaching adult levels at puberty—about 35 days.

The pattern of testis resembles that of liver rather than of pancreas. The enzyme seems to be associated with interstitial cells as deduced from several indirect experiments. The elevated temperature of the abdomen (23) causes germinal epithelium of the testis to disappear. In cryptorchid testes the concentration of esterase is increased above normal and only after 6 weeks—long after the germinal epithelium has disappeared—does the total esterase content fall. Hypophysectomy causes a profound decrease of testicular esterase; the injection of gonadotrophin, which stimulated Leydig cells of the testis, caused an increase of esterase. Gomori (18) found that in the rat testis only the in-

terstitial cells split glycol esters of stearic acid, germinal epithelium being devoid of "lipase." It should be pointed out, however, that in this, the histochemical method, liver, lung, and renal cortex show intense staining so that this reaction is interpreted by us as lacking specificity for lipase; non-specific esterase exhibits this hydrolytic phenomenon as well.

The evidence reveals further that the concentration of esterase in the testis of the rat is an indicator of the level of hormone production. The onset of pubertal growth of the prostate could be correlated (Fig. 2) with the increase of testicular esterase; the decrease of androgen production by the testis, as demonstrated by the weight of the ventral prostatic glands, was related to decreased testicular esterase, and its restoration by gonadotrophin caused an increase of esterase in the gonad.

SUMMARY

The tissues most effective in the enzymatic hydrolysis of acyl esters of *p*-nitrophenol by tissue extracts of the rat and dog were the liver, lung, pancreas, renal cortex, and testis; in the dog tracheal and vesical mucosae were also esterase-rich and the lens had appreciable activity. Esterase was at low concentration in the tissues of the rat for 4 and 5 days after birth, but an increase to adult levels soon took place except in the testis where the rise was delayed until puberty. The esterase values of the blood of newborn children were also low.

Two patterns of activity in tissues against these esters were found. A pattern in which propionate esters were hydrolyzed most rapidly was displayed regularly by liver, lung, renal cortex, and testis of the rat and also by dog, rabbit and human serum. A second pattern with progressive effectiveness in hydrolyzing fatty acid esters of 2-carbon to 5-carbon chain length was exhibited by the pancreas of the rat, rabbit, and dog and also by rat serum.

Esterase of the testis of the rat is located in the interstitial cells and its concentration seems to be directly related to androgenic hormone production. The increase of testicular esterase during puberty paralleled the increase of prostatic weight. Hypophysectomy caused a profound decrease of testicular esterase which was restored in part by gonadotrophin. Artificial cryptorchism in the rat, causing elimination of germinal epithelium, resulted in an increase of esterase concentration although the total content of the testis slowly decreased.

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THE OCCURRENCE OF NUCLEASES IN CULTURE FILTRATES OF GROUP A HEMOLYTIC STREPTOCOCCI

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The enzymes which are released into the environment by pathogenic microorganisms are of importance in a consideration of the mechanism of disease processes, since they may play a rôle in the virulence and invasiveness of the bacteria as well as in specific tissue injuries. In this connection, it is of interest that certain of the bacterial exotoxins have recently been demonstrated to be enzymatic in character. In the case of group A hemolytic streptococci, a number of extracellular products possessing a wide variety of biological activities have been recognized. Some of these substances, for example, streptolysin O, streptokinase, and streptococcal proteinase, have been subjected to intensive study, and a considerable body of information is available concerning their chemical and biological properties and their possible relationship to the pathogenesis of streptococcal disease. Because of the value of this type of study and the probability that other hitherto unrecognized substances accumulate in the environment of growing streptococci, the enzymatic activities of culture supernates of these organisms were investigated further. In the course of these studies, it was found that both ribonuclease and desoxyribonuclease occur with great regularity in culture media in which group A hemolytic streptococci are grown. The present paper deals with the extracellular occurrence and enzymatic activity of the two nucleases released from streptococcal cells during growth in fluid media.

Materials and Methods

Strains of Group A Hemolytic Streptococci.—Thirty-six different strains of group A hemolytic streptococci were studied for their ability to form nucleases. Sixteen serological types were represented and strains were included with special biological characteristics such as formation of large amounts of streptokinase or streptococcal proteinase, formation of hyaluronidase, failure to form erythrogenic toxin or streptolysin S, and requirement of the purine growth factor described by Wilson (1). A summary of the strains employed is given in Table I. The strains are designated by the numbers used in this laboratory, and the serological type is indicated in parentheses.

Bacteriological Media.—Three different media were employed: (a) Todd-Hewitt buffered broth (2), (b) neopeptone dialysate broth prepared according to the method of Dole (3), and (c) the partially defined medium developed by Adams and Roe for growth of pneumococci (4).

Preparation of Cultures.—16 to 18 hour cultures were used for the most part. The bacterial cells were removed by centrifugation, and in many experiments the supernates were tested without sterilization. While this procedure required that certain precautions be observed in

the handling and disposal of the material, it proved simpler than attempting to sterilize by filtration as a routine. However, it was found that the culture supernates could be passed through Coors P3 filters without loss of enzymatic activity, and this method was used to obtain bacteria-free filtrates.

Measurement of Ribonuclease.—A turbidimetric method was employed as the routine procedure for measuring ribonuclease activity, since the other methods available are not readily adaptable to use with nutrient broth. The method is based on the fact that under the

TABLE I

Strains of Group A Streptococci Tested for Production of Ribonuclease and Desoxyribonuclease

Special biological characteristics	Strain designation and serological type
Produces high yield of streptokinase	H105 (Tillett's "Co"; undesignated new type)
Produces high yield of streptococcal protease	B220 (Elliott's strain 5797; type 8 T antigen, no M antigen identified)
Produce extracellular hyaluronidase	C748 (type 4); B247 (type 22)
Stock strain for production of streptolysin O	D58 (Colebrook's strain "Richards," type 3)
Produce no streptolysin S	C439, C440, C441 (Colebrook's strains, all type 12)
Widely used for production of erythrogenic toxin	NY5 (type 12*)
Produces no erythrogenic toxin	C998 (Todd's "Cooper 3122," type 3)
Require Wilson's purine growth factor	C811, C812, C813, C272 (all type 19); C660 (type 19, no T antigen)
Do not require Wilson's purine growth factor	C817, C820, S24 (all type 19); C655 (type 19, no T antigen)
Recently isolated from scarlet fever patients	1GL19 (type 3); 1GL21 (type 17); 1GL4 (type 19); 1GL49 (type 30); 1GL22 (type 30)
Stock strains of various serological types	T1 (type 1); C203 (type 3, types 1 and 3 T antigens); S43 (type 6); S23 (type 14); J17E (type 17); J17F (type 26); T28 (type 28); D23 (type 29); D24 (type 30); London (undesignated new type)

* Originally classified as type 10 on the basis of typing by agglutination.

conditions employed the end-products of the reaction are soluble in acid, in contrast to the insolubility of the undigested nucleic acid. Ribonucleic acid from yeast was purified by the method of Kunitz (5). A stock solution containing 5 mg. per cc. was prepared by adding to an aqueous suspension of the nucleic acid just enough 1 N NaOH to cause complete solution. A solution so prepared has a pH of approximately 5.0 and is quite stable at refrigerator temperatures. A fivefold dilution of the stock solution in 0.025 M veronal buffer pH 7.5 is used as substrate for the test. The substrate solution is mixed with an equal volume of culture supernate and incubated in a water bath at 37°C. At intervals, 1.0 cc. of the reaction mixture is removed, mixed with 1.0 cc. of 1 N HCl, and the optical density of the resulting precipitate determined in a Coleman junior spectrophotometer at wave length 425 mμ. Turbidity controls for each of the reagents are employed. At appropriate enzyme concentrations the decrease in turbidity is linear with time over a period of 20 minutes. Contrary to expectation

on the basis of published data concerning the instability of yeast nucleic acid at alkaline pH, the substrate shows no spontaneous loss of acid precipitability in the absence of enzyme. Even after several hours at 37°C. and pH 7.5, the optical density after addition of HCl is identical with that of freshly prepared solutions.

Measurement of Desoxyribonuclease.—For routine testing of desoxyribonuclease a turbidimetric method identical with the ribonuclease method was used, except that the substrate solution was a 0.1 per cent solution of sodium desoxyribonucleate from calf thymus in 0.025 M veronal buffer pH 7.5 containing 0.01 M $MgSO_4$. In general, because of the higher activity of this enzyme, it was necessary to use dilutions of the culture filtrate to obtain linear rates of decrease in turbidity. Control tests of undigested substrate frequently give fibrous precipitates on the addition of acid which interfere with turbidimetric readings, but the results of enzymatic tests are unequivocal.

The turbidimetric method was supplemented by the more quantitative viscosimetric method previously described for estimating desoxyribonuclease activity (6). By this means, it was possible to make quite accurate comparisons of the potency of streptococcal nuclease with nuclease from other sources, e.g., beef pancreas.

EXPERIMENTAL

Nuclease Activity of Culture Supernates.—All thirty-six strains studied produced both ribonuclease and desoxyribonuclease, and no relation was detected to the other biological characteristics of the strains. Although there were quantitative differences in the amount of the two enzymes elaborated by different strains, the variations were not great and were not studied in detail. Supernates of cultures in neopeptone dialysate broth uniformly showed higher activity than those in Todd-Hewitt broth. This difference is not referable to greater growth of the organisms in dialysate broth, but is due, in part at least, to the presence of inhibitory substances in the Todd-Hewitt broth.

Ribonuclease activity was relatively low and dilution of the culture supernates was not necessary. The results of a test of a typical culture supernate are presented graphically in Fig. 1. The effect is comparable to that obtained with 0.01 $\mu g.$ of crystalline ribonuclease from beef pancreas as tested by the same method. In comparison with ribonuclease, desoxyribonuclease activity was surprisingly high. The data plotted in Fig. 1 show that a fiftyfold dilution of the culture supernate was required to give a decrease in acid precipitability comparable to that obtained with ribonucleic acid and undiluted culture supernate. The discrepancy in the relative activity of the two enzymes is probably even greater than that indicated, since the desoxyribonucleic acid has a much higher molecular size than the ribonucleic acid and presumably more enzymatic action is required to reduce the former to acid-soluble products. Evidence for this view was obtained in an experiment in which partial breakdown products of desoxyribonucleic acid, more nearly comparable in molecular size to the ribonucleic acid, were used as substrate in the turbidimetric test. At the same enzyme concentration, the rate of decrease in acid precipitability of the degraded substrate was four times as rapid as that of the intact nucleic acid.

The results of determination of desoxyribonuclease activity by the more

sensitive viscosimetric method confirm the findings of the turbidimetric test. With the same culture supernate as that employed in the test recorded in Fig. 1,

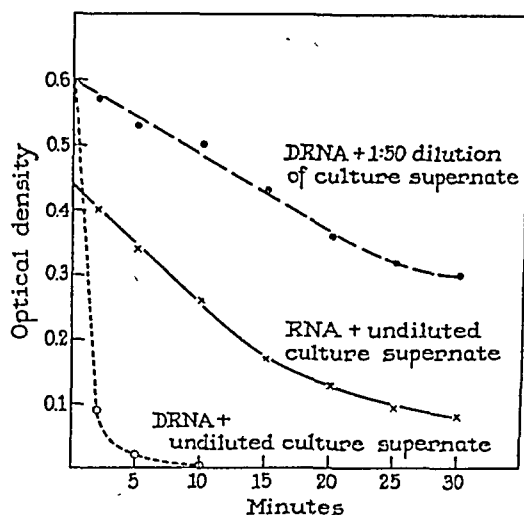


FIG. 1. Ribonuclease and desoxyribonuclease activity of culture supernate of strain C817 grown in neopeptone dialysate broth. DRNA indicates desoxyribonucleic acid, and RNA ribonucleic acid.

TABLE II
Relative Desoxyribonuclease Activity of Various Source Materials

Material	Units per cc.
Beef pancreas—0.25 N H ₂ SO ₄ or aqueous extract*	3300–3500
Swine pancreas—aqueous extract*	1000–2000
Group A hemolytic streptococci—culture supernate.	200–400
Type IIR pneumococcus—culture supernate.	1–2
Beef spleen—aqueous extract*.	<1
Calf thymus—aqueous extract*.	<1
<i>Escherichia coli</i> †—culture supernate.	0
<i>Bacillus subtilis</i> †—culture supernate.	0

* Tissue extracted with 2 to 3 volumes of solvent.

† One strain only tested.

0.0025 cc. (0.5 cc. of a 1:200 dilution) contains one unit of enzyme in the terms defined for use with the pancreatic nuclease (6). Thus, the unconcentrated culture supernate contains 400 units per cc., and a final dilution in the viscosimeter of 1:2000 causes a rapid fall in viscosity. With the exception of pancreatic extracts, streptococcal cultures have proved to be the most potent crude preparations of desoxyribonuclease of the various tissue extracts and bacteriological preparations tested in this laboratory. A comparison of the desoxyribonuclease activity of representative source materials is given in Table II.

It will be seen that the activity of the initial extracts of beef pancreas is about eightfold greater than that of streptococcal cultures. On the other hand, extracts of organs such as thymus and spleen have relatively little activity and the cultures of certain other species of microorganisms appear to be wholly devoid of desoxyribonuclease.

Rate of Production of Desoxyribonuclease.—Desoxyribonuclease is released into the medium by streptococci early in the active growth phase. This is demonstrated in Fig. 2, which records the results of a series of quantitative viscosi-

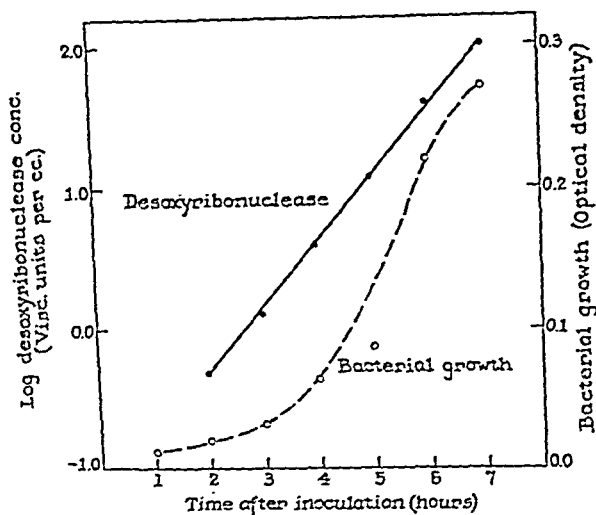


FIG. 2. Rate of increase in concentration of desoxyribonuclease in culture supernate during active growth of group A hemolytic streptococcus (strain H105).

metric determinations of desoxyribonuclease on samples of the supernate of the same culture at intervals after inoculation. A rather large inoculum of washed young cells (2×10^5 organisms per cc.) was used in order to avoid a long lag period. Desoxyribonuclease was detectable in the culture supernate at one hour and was present in accurately measurable amounts at 2 hours after inoculation. Bacterial growth was estimated turbidimetrically. As shown in Fig. 2, the concentration of enzyme increased logarithmically throughout the logarithmic growth phase of the organisms. It appears relatively certain, therefore, that the presence of the enzyme in the culture medium is not merely the result of the degeneration of aging cells.

Effect of Substrate on Nuclease Production.—An attempt was made to determine whether the presence of nucleic acids or enzymatic split products of nucleic acid influences the quantity of nuclease produced during growth. For this purpose it was necessary to use the partially defined medium of Adams and

Roe, since the more complex media contain unknown amounts of nucleotides. Most strains of group A hemolytic streptococci will not grow on repeated transfer in the Adams-Roe medium, but a few strains will give good growth provided moderately large inocula are used. A study of the growth of one strain (D24) by serial transfers in this medium revealed that both ribonuclease and desoxyribonuclease appear in the cultures, although in consistently smaller amounts than in the dialysate broth cultures.

The following substances were incorporated in the Adams-Roe medium at a final concentration of 0.1 to 1.0 mg. per cc.: desoxyribonucleic acid from calf thymus, ribonucleic acid from yeast, enzymatic split products of desoxyribonucleic acid prepared with pancreatic desoxyribonuclease, and enzymatic split products of ribonucleic acid prepared with crystalline ribonuclease. Strain D24 was grown in the presence of these various added substances, and in no case was there any measurable increase in nuclease content of the cultures as compared with control cultures in plain Adams-Roe medium. Furthermore, desoxyribonuclease production was not enhanced by the addition of large amounts of sodium desoxyribonucleate (up to 1 gm. per 100 cc. of culture) to cultures of streptococci in which heavy growth was obtained by continued neutralization of the acid formed in the presence of excess glucose. These experiments suggest that the nucleases, in contrast to streptococcal hyaluronidase (7), are not "adaptive" in character.

Relationship of Nuclease Production to Requirement for a Purine Growth Factor.—Wilson (1) has shown that certain strains of group A streptococci when inoculated into Adams-Roe medium supplemented with horse serum require the addition of a purine factor for growth. Viscous desoxyribonucleic acid did not serve as a source of the growth factor, although it was rendered active by digestion with pancreatic desoxyribonuclease. In a previous paper this finding was interpreted as indicating that these strains of group A streptococci lack the capacity to form an enzyme analogous to pancreatic desoxyribonuclease (6). However, the results of the present studies are not compatible with this interpretation, since several of the strains employed by Wilson have been tested and found not to differ from other strains with respect to desoxyribonuclease formation. Furthermore, a partially purified preparation of the enzyme was prepared from one of Wilson's strains (2884F, designated C811 in Table I) and was shown to be capable of converting calf thymus desoxyribonucleic acid into an active source of the growth factor, just as does pancreatic desoxyribonuclease. It is apparent, therefore, that the failure of the organisms to grow in the horse serum medium with added desoxyribonucleic acid is not referable to an inherent inability to form desoxyribonuclease. However, with the small inocula used (10^{-5} cc.), growth is probably not initiated in the absence of the purine factor and thus no enzyme is formed to convert the desoxyribonucleic acid into an available source of the factor.

DISCUSSION

The data recorded in the present paper indicate that both ribonuclease and desoxyribonuclease are produced by a wide variety of strains of group A streptococci during growth in fluid media. In relative terms, a large amount of desoxyribonuclease is formed; for example, 1 cc. of unconcentrated culture supernate is sufficient to cause depolymerization of several grams of desoxyribonucleic acid. Nothing is known at present concerning the possible rôle of the nucleases in the multiplication of streptococci in host tissues, and it has not been determined whether antibodies which inhibit the enzymes are formed following streptococcal infections. Because of the nature of their action, it seems unlikely that either of the nucleases is capable of damaging living cells, and their action *in vivo* is probably limited to the breakdown of nucleic acids released by tissue cells that have been destroyed by other agents.

The occurrence of the nucleases in culture supernates is not only of theoretical interest but has been shown to be of practical importance in the isolation and purification of other extracellular products, such as streptokinase and proteinase, since the nucleases must be separated from them in the course of purification procedures. Measurement of nuclease activity provides a delicate and simple test for the efficacy of the fractionation procedures employed. Furthermore, since streptococcal desoxyribonuclease is present in relatively large amounts, its preparation in purified form is feasible. In preliminary experiments fractions have been obtained which have a higher specific activity than pancreatic desoxyribonuclease made by the method previously described (6). These streptococcal desoxyribonuclease preparations have the additional advantage of being free of proteolytic activity.

The several enzymes which have been described as being released or "secreted" into the environment by group A hemolytic streptococci have in common the property of attacking substrates of large molecular size. Thus, streptokinase, streptococcal proteinase, hyaluronidase, ribonuclease, and desoxyribonuclease are involved directly or indirectly in the degradation of macromolecules. From one point of view, these enzymes may be interpreted as serving as a "digestive" function; that is, of preparing potential nutrient material so that it can be assimilated by the microorganism. Wilson's studies on the purine growth factor are in accord with this interpretation, since it seems apparent that the action of desoxyribonuclease on desoxyribonucleic acid results in the formation of certain products which the streptococcal cell is able to utilize. In connection with the possible "digestive" nature of these enzymes, it is of interest to compare the known enzymes of streptococcal supernates with those of mammalian pancreatic secretion. A single streptococcal culture supernate can contain a proteinase, a carbohydrase, and the two nucleases, enzymes which are representative of some of the main components of the pancreatic secretion. The analogy can be pursued further, since streptococcal proteinase,

like the pancreatic proteinases, has been shown to be released in the form of an inactive precursor (8).

SUMMARY

1. All of the thirty-six strains of group A hemolytic streptococci tested were found to elaborate ribonuclease and desoxyribonuclease during growth in liquid cultures. Both enzymes are released into the medium.

2. Desoxyribonuclease is consistently produced in greater amount than ribonuclease. The concentration of desoxyribonuclease in the culture increases logarithmically during active growth of the organisms.

3. Under the conditions employed, the presence of specific substrate or enzymatic split products of the substrate did not influence the production of either nuclease.

4. The failure of viscous desoxyribonucleic acid to serve as a source of the purine growth factor required by certain strains of group A streptococci was shown not to be referable to the inability of these strains to form desoxyribonuclease.

5. The determination of nuclease activity provides another criterion for evaluating purification procedures used in the attempted isolation of substances such as streptokinase and proteinase from the supernates of streptococcal cultures.

The author takes pleasure in acknowledging the able technical assistance of Miss Elizabeth Van Pelt.

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A MEANS OF INCREASING THE TUBERCULOSTATIC EFFECT OF KNOWN CHEMOTHERAPEUTIC AGENTS

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Dubos and his coworkers recently have shown the profound influence that certain surface-active oleates have on the *in vitro* growth of the tubercle bacillus (1-3). Their work suggests that by the addition of these fatty acid in a highly surface-active form a great enhancement of growth can be obtained. The mechanism of action of this surface-active growth-promoting factor has been interpreted according to known physical-chemical principles, which would indicate that the oleate is concentrated at the periphery of, or within the confines of the mycobacterium.

In the study here to be reported,¹ several surface-active tuberculostatic substances have been prepared and tested in an effort to determine whether a similar increased effect on the tubercle bacillus would be obtained by concentrating an inhibitory instead of a stimulating substance about the organism. These substances were found to be effective *in vitro* in relatively low concentrations.

The mechanism of such tuberculostatic action was investigated according to physical-chemical principles, by isolating the factor of drug action due to its surface activity from its inherent (non-surface-active) component. This was carried out by introducing a substance antagonistic to the surface-active component of the drug, but in no other way interfering with its antibacterial action. These studies suggest that the property of surface activity may enhance the tuberculostatic action of a given drug. The reason for such action is discussed and correlated with the steric arrangement of the drug molecule in relation to the surface of the mycobacterium. Other previously described surface-active tuberculostatic chemicals and antibiotics have also been studied with this concept in mind, and certain of them are shown to owe a part of their activity to the surface-active character of their molecules.

Hypotheetical Effect of Surface-Active Substances upon Mycobacteria.—In general, studies of the effects of surface-active substances upon the growth of bacteria and of acid-fast organisms in particular (4-8) have emphasized the fact that human tubercle bacilli can grow *in vitro* at a vastly reduced surface

¹ I wish to thank Dr. Evarts Graham, Dr. W. Barry Wood, and Dr. J. Bronfenbrenner for their help and assistance throughout the planning and execution of this study.

tension, most authors agreeing that growth is possible at 35 to 40 dynes per sq. cm. It was shown that under such conditions the growth of the organisms which normally occurs as a surface pellicle would submerge in liquid medium. Other characteristics of bacteria were also noted in the presence of surface-active substances, such as variations in pathogenicity (8), and the anti-swarming effect on *B. proteus* (9). Stanley (10) has commented upon the correlation between known leprocidal agents, and their ability to lower surface tension, suggesting that the "soapiness" of solutions of these chemotherapeutic agents might in some way account for their bacteriostatic action.

Dubos (1) in 1945 first showed the remarkable stimulating effect that water-soluble lipids in the form of a surface-active agent could have on the *in vitro* growth of the tubercle bacillus. He used a phospholipid (which is a naturally

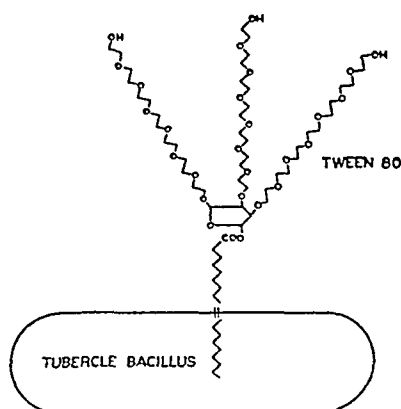


FIG. 1. Proposed orientation of Tween 80 molecule upon surface of tubercle bacillus

occurring surface-active substance) as well as a synthetic commercially available surface-active agent, Tween 80. The addition of such a surface-active agent in concentration of 50 mg. per cent reduced the time required for growth in a liquid medium to less than a week. Many surface-active substances were tried before tests led to the choice of Tween 80. In particular Dubos (11) has shown that the stimulating effect is inherent in the hydrophobic end of the Tween 80 molecule, which is an oleate. The remaining portion of the molecule is merely a water-solubilizing complex, which in the case of Tween 80 consists of a sorbitan nucleus attached to three polyethylene glycol chains.

If the known properties of surface-active substances be applied to the Tween 80 molecule, its steric arrangement about the tubercle bacillus can be illustrated as in Fig. 1. This surface-active molecule will concentrate around the water-bacillus interface, with the hydrophobic portion of the molecule (the oleate) placed toward or pushed within the bacterium. The exact chemical nature of the structures on the periphery of the tubercle bacillus is under critical consid-

eration at the present time. Whether a true fatty capsule, a lipid layer, or a lipoprotein complex exists is debatable (12), but as far as this study is concerned is of little consequence. Whatever its nature, at least an interface is formed which on one side is aqueous and on the other is high in lipid content (13). It is at this interface that the surface-active molecules adlineate. Whether actual penetration of this lipid layer is attained is only problematic.

One of the actions of the Tween 80 molecule in Dubos' media therefore may be that of concentrating a known metabolite (the oleate) at a point where it would be most readily available to the organism, namely at the periphery of, or possibly within the confines of the bacterial cell. By dispersing the normally clumped tubercle bacilli, Tween 80 asserts another type of stimulating effect upon bacterial growth.

Instead of concentrating an essential metabolite at the tubercle bacillus boundary, as in the case of Tween 80, it was anticipated that a known tuberculostatic substance might be used as the hydrophobic end of a surface-active molecule. In this way the effective concentration of the drug might be increased, for the surface-active molecules instead of being spread evenly throughout the solution would adlineate about the periphery of the bacteria. Many compounds have been shown to be effective *in vitro* against the tubercle bacillus (14). Of these, among the best known and most thoroughly investigated are the diaminodiphenyl sulfone derivatives. Promin, promizole, and diasone are some of the derivatives that have been produced commercially from this mother compound. Because of the availability of this compound, its rather simple chemistry, and because of its water-insoluble nature, diaminodiphenyl sulfone was used as the tuberculostatic compound with which to test this hypothesis of drug action. By attaching a water-soluble component to one end of the diaminodiphenyl sulfone in such a way as to solubilize in a balanced manner the molecule, a surface-active drug fitting the requirements could be obtained. Fig. 2 *a* illustrates a surface-active oleate, such as would enhance the growth of the tubercle bacillus. Figs. 2 *b* and 2 *c* represent an analogous surface-active sulfone which could act in a like manner to inhibit mycobacterial metabolism. In order to assure the orientation of the drug molecules, some of the drugs were compounded with the active sulfone attached to the end of an already surface-active molecule, as illustrated in Fig. 2 *c*.

Investigation of Mechanism of Drug Action.—If the principles of drug action upon which these materials were prepared were correct, the surface-active agents should assert a tuberculostatic effect at a relatively low concentration. Mere measurement of antibacterial activity is, however, no proof of the mode of antibacterial action. For this reason, a means had to be found to isolate and measure that component of drug action which was dependent solely upon the surface-active properties of the drug. Such a method would determine whether

molecular orientation of a tuberculostatic drug about the mycobacteria could enhance its antibacterial effect. If such an increase of effect were noted, the validity of the hypothetical mechanism of drug action would be supported.

In order to determine the increase in drug action due to its surface activity, recourse was had to another basic physical-chemical principle. If two surface-active substances are in an aqueous system, the more highly surface-active compound will crowd the less active substance from the interface of such a system. Applying this principle to the problem at hand, a more powerful surface-active substance in the form of Tween 80 could be added to the medium

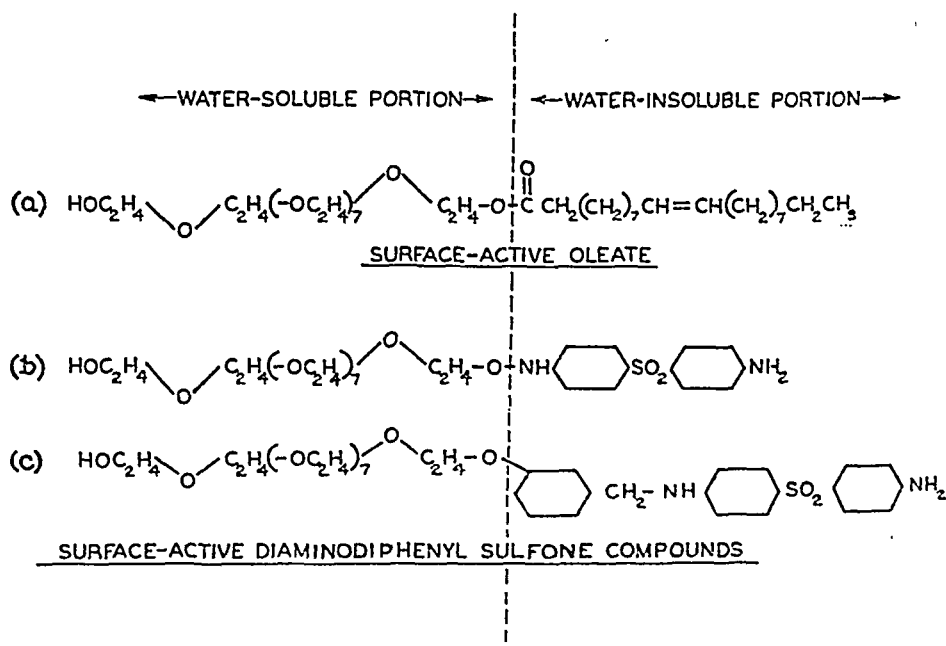


FIG. 2

in order to crowd the less active drug away from the periphery of the tubercle bacillus. The surface-active sulfones would be adsorbed around the acid-fast organisms, but when Tween 80 was added the sulfones would be displaced from this interface, and would therefore lose the component of drug action due to their molecular orientation. In this way the component of drug action dependent on surface activity could be selectively abolished and thereby accurately measured. Non-surface-active drugs, on the other hand, would in no way be inhibited by Tween 80, thus furnishing an experimental control in the evaluation of drug action.

EXPERIMENTAL

Method in Determining Tuberculostatic Potency of Drugs.—Serial dilutions of a number of surface-active sulfones in appropriate liquid media were made in order to determine the min-

imum concentration of drug required to inhibit the growth of various types of mycobacteria.

A number of types of mycobacteria were tested in conjunction with the synthesized drugs as well as with controls. These ranged from the saprophytic *Mycobacterium tuberculosis* 607 to the virulent human H37Rv strain. These organisms included:²

Mycobacterium tuberculosis H37Rv

Mycobacterium tuberculosis H37Ra

Mycobacterium tuberculosis var. *hominis* 607

Mycobacterium avium

Mycobacterium tuberculosis var. *bovis*

Mycobacterium phlei

Mycobacterium stercois

As the work with these surface-active materials progressed, it was found that, in order to avoid errors in titer of drug action, a medium containing a minimum of surface-active material was required. For this reason the Verwald modification of Proskauer-Beck synthetic medium was used at pH 7.2.

Inoculations of mycobacteria other than the H37 strain were made with one loopful of a 72 hour growth. The organisms were incubated at 37°C. for 72 hours before being read.

The H37 strain was grown on Dubos' albumin-oleate medium (15). Inoculations were made with approximately 0.01 mg. of the organisms, the virulence of which had been maintained by mouse passage. Incubation was at 37°C. for 10 days. There usually was no difference in readings between the 6th and 10th days, but the longer period was used as final.

A series of surface-active sulfones was prepared by the Monsanto Chemical Co. according to the principles discussed above.³ In order to make the surface-active properties of the drugs independent of pH variations, non-ionic or very slightly ionizing compounds were used.

Table I shows the chemical nature and formulae of the surface-active drugs used, as well as the control drugs included for study. All the compounds except diaminodiphenyl sulfone were water-soluble and were added in aqueous solution in the proper dilutions to the media. Ethylene glycol was used to dissolve the water-insoluble 4,4'-diaminodiphenyl sulfone.

Surface tension determinations were made with a du Noüy ring tensiometer. The values for these determinations are given in Table II. It is obvious from these determinations that the inhibitory effect of the drugs upon the growth of the tubercle bacillus is not simply by means of lowering the surface tension of the medium, for in no case was the surface tension at the level critical for mycobacterial growth, namely 35 to 40 dynes per sq. cm.

Included in this study was pyrididin (sodium formaldehyde bisulfite of 5-amino-2-butoxypyridine), a tuberculostatic agent recently described by Feinstone (16).

Results of Tuberculostatic Measurement.—In general, the bacteriostatic efficiency of each drug was similar when tested with the various types of mycobacteria. This finding is in agreement with previous studies (16, 17). For this reason, only the results obtained with the virulent H37Rv human strain are given, even though all the other mycobacteria were tested.

² The strains of *Mycobacterium tuberculosis* H37Rv and H37Ra were kindly supplied by Mr. W. Steenken, Jr., from the Standard Culture Depot of the National Tuberculosis Association, Trudeau. The other strains of mycobacteria were obtained from the American Type Culture Collection.

³ It is a pleasure to acknowledge the help of Mr. Paul Logue, Dr. Paul Glusenkamp, and Mr. Milton Kosmin, all of the Monsanto Chemical Co., in the chemical problems involved in this study. The surface-active sulfones were synthesized under their direction at the Central Research Department, Dayton.

TABLE I

No.	Name	Chemical formula	Chemical nature
1	Sterox 1	$\text{HOC}_2\text{H}_4-(\text{OC}_2\text{H}_4)_2-\text{OC}_2\text{H}_4-\text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2$	4,4'-Diaminodiphenyl sulfone ethylene oxide condensation product
2	Sterox 2	$\text{HOC}_2\text{H}_4-(\text{OC}_2\text{H}_4)_{11}-\text{OSO}_2\text{H} \cdot (2)\text{NH}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NH}_2$	Carbowax 600 sulfate of 4,4'-diaminodiphenyl sulfone
3	Sterox 3	" "	Same as sterox 2 but with less sulfone (50 per cent less of theoretical)
4	Sterox 4	$\text{HOC}_2\text{H}_4(\text{C}_2\text{H}_4\text{O})_9\text{HN} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NHC}(\text{CH}_2)_6\text{CH}_3$	4-Capryloylamino-4'-aminodiphenyl sulfone plus ethylene oxide
5	Sterox 5	$\text{HOC}_2\text{H}_4(\text{C}_2\text{H}_4\text{O})_9\text{OC}_2\text{H}_4\text{O} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{CH}_2-\text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NH}_2$	Condensation of chloromethyl triton N/100 with 4,4'-diaminodiphenyl sulfone
6	Sterox 7	$\text{HOC}_2\text{H}_4(\text{C}_2\text{H}_4\text{O})_9\text{OC}_2\text{H}_4\text{O} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{CH}_2-\text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{HOC}_2\text{H}_4(\text{C}_2\text{H}_4\text{O})_9$	Same as sterox 5 but with added ethylene oxide
7	Sterox 6	$\text{HOC}_2\text{H}_4(\text{C}_2\text{H}_4\text{O})_9\text{C}_2\text{H}_4\text{O}-\text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{N}-\text{CH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{OH}$	Isovanillin anil of 4,4'-diaminodiphenyl sulfone
8	Sterox 8	$\text{HOC}_2\text{H}_4(\text{OC}_2\text{H}_4)_{11}-\text{OC}_2\text{H}_4\text{O} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{O}-(\text{C}_2\text{H}_4\text{O})_{11}-\text{C}_2\text{H}_4\text{OH}$	Condensation product of 2,4'-dihydroxy diphenyl sulfone plus ethylene oxide
9	Sterox 9	Same as above except a total of 20 mols of ethylene oxide instead of 24' and a mixed isomer used	Condensation product of mixture of 2,4'- and 4,4'-dihydroxy diphenyl sulfone with ethylene oxide
10	Sterox 10	$\text{HOCH}_2\text{CH}_2-(\text{OC}_2\text{H}_4)_{11}\text{OC}_2\text{H}_4\text{SO}_3\text{H} \cdot \text{NH}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NH}_2$	4,4'-Diaminodiphenyl sulfone salt of polyethylene glycol sulfonic acid
11	*	$\text{NH}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NH}_2$	4,4'-Diaminodiphenyl sulfone
12	Promin*	$\text{HOCH}_2(\text{CHOH})_4-\text{CH}-\text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{SO}_2 \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{CH}-\text{NH} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{HOCH}_2(\text{CHOH})_4$	Sodium salt of 4,4'-diaminodiphenyl sulfone N,N' di- (dextrose sulfonate)
13	Pyricidin†	$\text{C}_6\text{H}_5\text{O} \begin{array}{c} \diagup \text{C}_6\text{H}_4 \diagdown \\ \diagdown \text{C}_6\text{H}_4 \diagup \end{array} \text{NHCH}_2\text{SO}_2\text{Na}$	Sodium formaldehyde bisulfite of 5-amino-2-butoxypyridine

* Kindly supplied by Parke, Davis and Co., Detroit.

† Kindly supplied by the Pyridium Corporation, Nepara Park, Yonkers.

The remainder of the drugs were all synthesized by the Central Research Department of the Monsanto Chemical Co., Dayton.

As can be seen from Table III, the drugs vary widely in their tuberculostatic effect. Most of the compounds are tuberculostatic in very low concentrations,

TABLE II
Surface Tension of Drugs in Solution

Drug	Surface tension 10 mg. per cent aqueous solution
	<i>dynes/cm.</i>
Water.....	72
Sterox 1.....	60
Sterox 2.....	59
Sterox 3.....	65
Sterox 4.....	45
Sterox 5.....	42
Sterox 6.....	53
Sterox 7.....	44
Sterox 8.....	59
Sterox 9.....	56
Sterox 10.....	51
Promin.....	72
Pyricidin.....	55

TABLE III
Tuberculostatic Effect of Drugs

Drug	Minimum concentration inhibiting growth of <i>Mycobacterium tuber-</i> <i>culosis</i> H37Rv
	<i>mg. per cent</i>
Sterox 1.....	20
Sterox 2.....	25
Sterox 3.....	1.0
Sterox 4.....	>20
Sterox 5.....	0.07
Sterox 6.....	>20
Sterox 7.....	1.5
Sterox 8.....	>20
Sterox 9.....	>20
Sterox 10.....	0.6
Promin.....	65
Diaminodiphenyl sulfone.....	0.6
Pyricidin.....	0.06

and are effective in ranges well below that of promin. Though the absolute degree of effectiveness is not of major importance in supporting the hypothesis of drug action, these compounds are definitely potent tuberculostatic substances.

Certain of the compounds are seen to be less active than others. In those cases in which ethylene oxide was added in an effort to make the compound more water-soluble, a decrease in tuberculostasis is noted. This is felt to be due to the fact that the previously unbound amino group on the sulfone is tied up by the additional ethylene oxide. As has been demonstrated with the sulfonamides (18), such a condition decreases bacteriostatic activity, since a free amino group seems to be required for optimum action.

In like manner, those compounds in which the sulfone is in the middle of the molecule—sterox, 1, 4, and 6—are of less absolute tuberculostatic value. This may be due to the absence of a free amino group, as when ethylene oxide is added. On the other hand, such a molecule would not have the active sulfone at the end of the hydrophobic portion of the molecule and thus might be utilized less readily by the organism.

Two compounds—sterox 8 and 9—were made in which dihydroxy diphenyl sulfone was used as the base, but neither of these substances was as effective as the diamino derivative.

The most active of these compounds are those in which orientation of the molecule by means of its surface activity is assured. Sterox 3, sterox 5, and sterox 10 are all effective against the mycobacteria, all have definite surface-active properties as gauged by their surface tension depressant action, and all have the sulfone on one end of the molecule. These are conditions that, according to the theory of drug action, might be required for most efficient action.

The potency of pyridicin against the tubercle bacillus as stated by Feinstein (16) is confirmed in this study.

Method in Evaluating Component of Drug Action Due to Its Surface Activity.—In order first to determine the qualitative effect of the highly surface-active Tween 80 upon the tuberculostatic action of the drugs, the minimum concentration inhibiting mycobacterial growth was determined in the presence of and in the absence of Tween 80. This was accomplished by inoculating *Mycobacterium tuberculosis* var. *hominis* 607 into tubes of Proskauer-Beck medium containing graded dilutions of the surface-active tuberculostatic agents. Another comparable series was run with graded drug dilutions plus 0.05 per cent Tween 80 added to the medium. A comparison of the tuberculostatic activity in the presence of and in the absence of Tween thus indicated the presence or absence of interference in drug activity.

When large amounts of surface-active substances were present in the media, the organisms grew not as a surface pellicle, but as a submerged layer, or diffusely throughout the culture tube. End-point determinations for inhibition of growth continued to be clear-cut, however.

So that a more quantitative measure of this antagonistic action between the surface-active drugs and the more highly surface-active Tween 80 could be made, tests of drug activity in the presence of varying concentrations of Tween 80 were carried out.

A graded series of drug dilutions were added to a similar set of Tween 80 dilutions so that the amount of drug required for mycobacterial inhibition could be determined for varying concentrations of Tween 80. Again *Mycobacterium tuberculosis* var. *hominis* 607 was used as the test organism in Proskauer-Beck medium. As indicated in Fig. 3, two of the surface-active sulfones (sterox 3 and 5) as well as pyridicin were tested in the presence of varying concentrations of Tween 80. The end-point in each series of dilutions was the minimum concentration

of drug that would inhibit the mycobacterial growth in the presence of the given Tween 80 concentration.

This type of experiment is similar to that carried out by Wood (19) in which the antagonistic action between the sulfonamides and paraaminobenzoic acid was tested. In the case of the surface-active sulfones, however, their inhibition is only partial, for but one component of drug action is neutralized, namely that portion due to its surface activity.

Results of Tuberculostatic Inhibition.—Table IV shows the results of the first series of experiments in which a given concentration (0.05 per cent) of

TABLE IV

Difference in Tuberculostatic Effect in the Absence and in the Presence of 0.05 Per Cent Tween 80

Number	Name	Per cent active principle of total molecular weight	Concentration inhibiting growth of <i>Mycobacterium tuberculosis</i> var. <i>hominis</i> OGI		Difference in activity when surface action of drug is abolished by Tween 80
			Proskauer-Beck medium	Proskauer-Beck medium plus 0.05 per cent Tween 80	
		per cent	mg. per cent	mg. per cent	
1	Sterox 1	36	1.5	200	130 ×
2	Sterox 2	27	0.9	150	166 ×
3	Sterox 3	17	0.4	200	500 ×
4	Sterox 4	19	>20	200	
5	Sterox 5	26	0.2	75	375 ×
6	Sterox 6	37	>20	200	
7	Sterox 7	18	0.5	150	300 ×
8	Sterox 8	19	>20	200	
9	Sterox 9	22	20	200	
10	Sterox 10	28	0.75	150	200 ×
11	Diaminodiphenyl sulfone	100	0.072	0.072	None
12	Promin	31	4.0	5.0	Negligible
13	Pyrididin	100	0.062	25	403 ×

Tween 80 was used to determine qualitatively whether any inhibition in drug effect was attained by the highly surface-active oleate. As can be seen, a great discrepancy exists between the tuberculostatic powers of the surface-active sulfones in the absence of and in the presence of the more highly active, and growth-promoting Tween 80. In the case of sterox 3 there was a decrease of 500 times in drug activity when 0.05 per cent Tween 80 was present. By partially interfering with the molecular orientation of the drug about the bacteria, therefore, the activity of the drug was decreased 500 times. Stating the situation in a reverse manner, it could be said that making the drug surface-active with its resultant steric arrangement about the organism, increases its tuberculostatic action 500 times.

It is quite evident from these data that no appreciable inhibitory effect is had

upon those drugs that are not in themselves surface-active. Diaminodiphenyl sulfone, and promine are not affected, whereas the various surface-active sulfones and pyricidin are markedly inhibited in their bacteriostatic effect by these surface-active antagonists. It would seem that this furnishes a method of differentiating the mechanism of drug action between those compounds which rely for their action to some extent on their surface activity from those compounds which do not have this physicochemical property as a basis of their activity

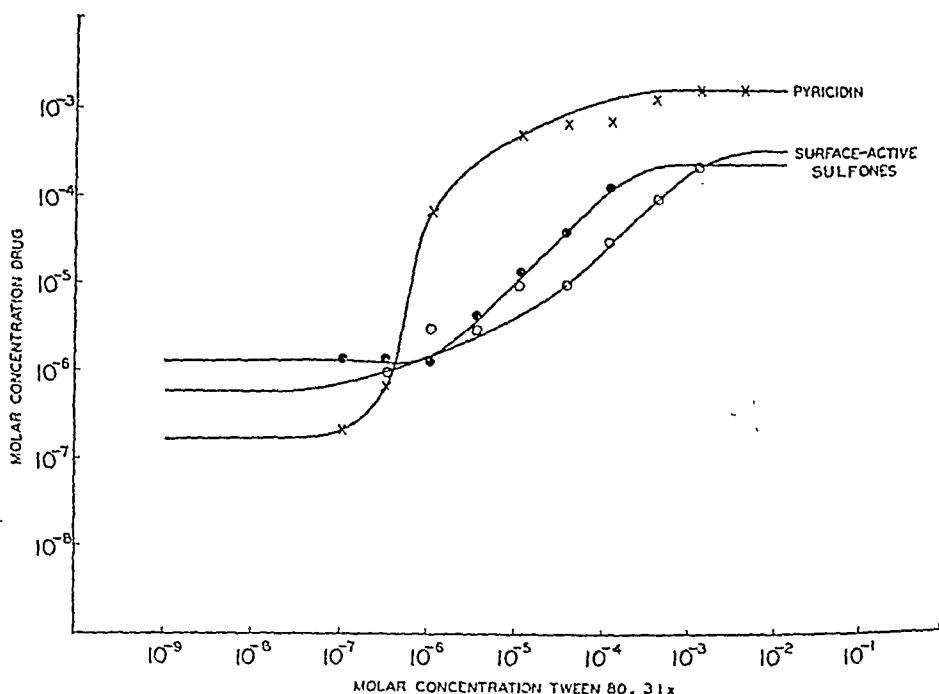


FIG. 3. Inhibition of tuberculostatic action of surface-active drugs by more highly surface-active Tween 80.

More precise and quantitative measurements of this tuberculostatic antagonism are recorded in Fig. 3. In this experiment the two most effective surface-active sulfones (sterox 3 and 5) as well as pyricidin were tested in the presence of varying concentrations of Tween 80. The tuberculostatic endpoints in this experiment are plotted as a function of the Tween 80 and drug concentrations.

In very low concentrations of Tween 80 there is no inhibiting effect upon drug action—the flat part of the curve where a minimum of drug was needed to cause bacteriostasis. As more Tween 80 was added, however, an increased amount of drug was required to inhibit bacterial growth. At a certain point the addition of more Tween 80 caused no further diminution in the effect of the drug, and at this point the curve leveled off.

Non-surface-active drugs are not antagonized in their bacteriostatic action by Tween 80. Graphically, such substances as promin, diaminodiphenyl sulfone, and sodium azide produce a straight horizontal line in experiments such as that illustrated in Fig. 3 for their action is independent of Tween 80 concentration. At that point where the steric concentration component of the surface-active drugs is abolished, they too act like non-surface-active drugs, thus accounting for the leveling of the curve in Fig. 3.⁴ Returning to the physical-chemical principles involved, the antagonistic action between the two surface-active substances in the form of Tween 80 and the surface-active drugs, may be pictured as a competition for a place at the mycobacterial boundary. Increasing concentrations of Tween push the less surface-active drug from the bacterial cell back into the surrounding medium. At a certain point, all of the surface-active drug would be pushed from this interface and further addition of Tween 80 would assert no more effect. At this point the curve flattens out on the graph, for the surface-active component of the drug has been entirely abolished and its action is that of any non-surface-active agent: it is bacteriostatic when metabolized by the organism, but has no particular steric adlineation around the bacillus.

DISCUSSION

The Surface-Active Component in Some Previously Described Bacteriostatic Substances.—In support of the mode of drug action described above, several drugs previously reported in the literature can be cited (22). If molecular orientation about an organism by reason of surface-active properties enhances the bacteriostatic effect of a drug, then drugs having any degree of such surface activity should demonstrate the principle involved. Such drugs were found and their mode of action proven to be similar to that of the synthesized surface-active sulfones.

Feinstone (16) recently has introduced a new series of potent tuberculostatic substances. He discussed the efficiency of such substances but has not suggested the mode of action of the compounds. The drug showing the greatest promise, according to the author, was sodium formaldehyde bisulfite of 5-amino-2-butoxypyridine. This drug has been called pyricidin by its manufacturer. Examination of the chemical nature of this drug (Table I) suggests that the compound would have surface-active properties, and it does, as a matter of fact, lower surface tension of an aqueous solution markedly, as shown in Table II.

Just as the surface-active sulfones were described as concentrating about the mycobacteria, so the surface-active pyricidin molecules can be pictured at the bacterial interface. In the latter case, an entirely different hydrophobic and

⁴ Far from being antagonized, the action of some non-surface-active bacteriostatic substances is enhanced by the addition of surface-active agents. This is probably due to the dispersing and wetting power of the capillary-active material (20, 21).

tuberculostatic material is concentrated around or within the cell, consisting of a pyridine nucleus instead of a sulfone. Though the basic mode of metabolic inhibition with the two drugs is probably very different, they are concentrated about the organism in a similar fashion—by means of their surface-active properties.

That pyridicin does indeed owe some of its activity to its molecular concentration about the bacteria is shown in the similarity of its experimental behavior to that of the surface-active sulfones. This is evident from examination of Fig. 3 and Table IV. The bacteriostatic activity of both pyridicin and the surface-active sulfones is diminished when their steric arrangement in regard to the bacterial boundary is disturbed. Under these conditions, both groups of drugs maintain a bacteriostatic effect, but one which is much less than is the case when their surface-active properties are allowed to come into play. Both therefore appear to have their antibacterial action enhanced by reason of their surface activity.

Another group of tuberculostatic substances that have surface-active properties has been introduced by Freedlander (23). This author has studied the chemotherapeutic properties of certain sulfonium and iodonium salts which he has noted in passing are to some degree surface-active. He further states that, though the sulfonium and iodonium compounds belong to the class of cationic surface-active substances, their action is not that of the commonly used germicidal detergents. It would seem that the mechanism of action of this group of tuberculostatic agents might in part be explained by their property of surface activity, as in the case of pyridicin.

Certain of the antibiotics also are found to be surface-active, and their activity must be considered in the light of the physical-chemical principles herein described. Gramicidin (24) and its various breakdown products (25, 26) have marked surface-active properties. The antibiotic subtilin is likewise reported to be surface-active (27). Although the exact chemical nature of these antibiotics is not known, the surface-active molecules may be pictured as concentrating about an organism in a manner analogous to that of the chemically simpler surface-active drugs.

Surface-Active Bacteriostatic Agents in Contrast to the Detergents.—Since Domagk (28) first observed the bactericidal action of certain cationic detergents, a vast literature on this subject has accumulated. Many cationic surface-active agents are commercially available (zephiran, phemerol, cecpryn, etc.) and have found wide use as topical germicides. They have unfortunately a non-specific cytolytic action, effectively disintegrating not only bacteria, but tissue cells as well. For this reason their systemic use is precluded.

The surface-active bacteriostatic agents used in this study owe their essential antibacterial activity to properties other than mere cell destruction. Their mode of action is essentially the same as that of the chemotherapeutic mother

compound from which they are derived—in the case of the drugs used in this study, that of the sulfone. They function therefore as selective inhibitors, blocking enzyme systems that are vital for bacterial growth, but are relatively less important to tissue cell metabolism. The surface-active properties of such drugs effectively increase their apparent antibacterial activity but they continue to operate in a manner which makes them more toxic to bacteria than to host cells. The active hydrophobic end of the surface-active drug may approach many cells but is relatively toxic only to the bacteria. Instead of being classed with the cationic detergents as general protoplasmic poisons, therefore, these substances may be considered chemotherapeutic agents.

Bactericidal-Wetting Agent Mixtures.—Simple mixtures of various types of bactericidal and bacteriostatic drugs with surface-active substances have been investigated by many authors (21, 29–33). The consensus of opinion has been that a potentiation of bactericidal effect is obtained by means of these mixtures (20). The mechanism of such action is felt to be due to the wetting action of the detergent upon the bacteria, thus sponsoring closer contact between drug and organism (34).

The use of mixtures of drugs and wetting agents is, of course, a condition different from the employment of a single molecular compound containing both bacteriostatic and surface-active properties. Wetting of the bacteria may not necessarily produce closer contact between drug and organism, for the bacteriostatic agent is in no way attached to the surface-active molecule that coats the bacteria. Contact between drug and bacteria would in this case depend upon the interaction between the drug and the wetting agent, as well as on the nature of the organism involved. If, on the other hand, one molecule combined the two characteristics of surface activity and bacteriostatic action, such condition of contact is assured.

Applications of These Principles of Drug Activity.—If these principles of drug activity should be confirmed, their application to various types of chemotherapeutic agents should be feasible. Surface-active properties can be relatively easily imparted to many types of molecules. In the case of antibacterial drugs, this of course may change the essential metabolic activity of the compound, and this effect would have to be considered in drug synthesis.

Because of their high lipid content, only mycobacteria have been included in this study. The physical-chemical principles involved, however, are equally applicable to other types of organisms, though according to Feinstone's results with pyridin (16), the drug is much less effective against organisms other than mycobacteria.

Since the surface-active properties of these drugs need be relatively slight in order to assure their molecular orientation, their toxicity in this respect may not be great. The bacterial inhibition that they induce is not dependent solely on their action as a surface tension depressant, and therefore they need have

none of the cytolytic properties of the strong detergents. *In vitro* experiments with the drugs used in this series, for example, demonstrate that many times the concentration required for tubercle bacillus inhibition is necessary to initiate hemolysis.

SUMMARY AND CONCLUSIONS

By incorporating a known tuberculostatic agent on one end of a surface-active molecule, an increased *in vitro* effect on the tubercle bacillus has been obtained.

This activity is presumably due to the concentration of the drug molecules at or beneath the mycobacterial cell boundary.

Whether the surface-active drug actually penetrates the lipid, or lipoprotein complex that is so characteristic of the mycobacteria is only problematic. According to comparable purely physical-chemical experiments, such a penetration is quite likely.

Increase in tuberculostatic effect of over 1000-fold has been attained by rendering surface-active the drugs included in this study. The quantitative evaluation of this increase in activity has been obtained by measuring drug action both when its surface-active properties are functional, and when these properties have been selectively abolished by a specific surface-active antagonist.

It is believed that the molecular orientation of a surface-active drug about a bacterial cell accounts for one component of its antibacterial action. Certain previously described surface-active antituberculous drugs and antibiotics have been examined in light of this interpretation.

It may be anticipated that the more potent tuberculostatic drugs such as streptomycin could be made more effective by incorporating the molecule into a surface-active compound, according to the principles herein described.

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THE IMMUNOCHEMISTRY OF TOXINS AND TOXOIDS

VI. THE CRYSTALLIZATION AND CHARACTERIZATION OF TETANAL TOXIN*

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It is desirable to obtain all functional proteins in a pure state to study their nature, properties, and mode of action. This is especially true for bacterial toxins. At present, little or nothing is known concerning the primary chemical or physiological effects of bacterial toxins; even the tissue cells affected are for the most part unknown. Thus, information on either the nature or the mode of action of bacterial toxins would permit a better understanding of the specific physiologic functions which are disturbed during the course of infections and would perhaps suggest improved immunotherapeutic or specific chemotherapeutic measures.

Several exotoxins have been isolated in highly purified form. Both Eaton (1) and Pappenheimer (2) have prepared purified diphtherial toxin. Two groups (3, 4) working at Camp Detrick have reported the crystallization of *botulinum* toxin (type A). Scarletinal toxin (5) and streptolysin (6) have been highly purified. Although these toxins have been characterized as heat-labile proteins, physical and chemical analyses have as yet offered no explanation for their extreme toxicity.

Pickett, Hoeprich, and Germain (7) prepared tetanal toxin (Mueller) in a highly purified state by neutral salt precipitation and adsorption on cadmium compounds. Their best preparation contained 0.023×10^{-6} mg. N per m.l.d. which represented a product of about 100 times greater purity than had been previously reported (8). These authors made no claim that they had reached the limits of purity for this toxin.

In a preliminary communication, Pillemer, Wittler, and Grossberg (9) reported the crystallization of tetanal toxin. The purpose of the present paper is to describe in detail the methods for the purification and crystallization of tetanal toxin. Certain chemical and physical properties of the crystalline toxin are also presented.

EXPERIMENTAL

Materials.—The parent tetanal toxins¹ employed in this study were prepared according to the method of Mueller and Miller (10) and contained between 20 and 40 Lf units and between

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¹ Supplied by Lederle Laboratories and Wyeth, Inc.

200,000 and 500,000 M.L.D. per ml. It is doubtful whether the present study could have been carried to completion if this toxin had not been available, because attempts to crystallize tetanal toxins prepared on veal infusion-proteose-peptone medium had not been successful.

All chemical reagents were either c.p. or the best grade obtainable. The acetate buffer used for pH adjustments was composed of 2 M acetic acid and 0.4 M sodium acetate. Diluted 40-fold with distilled water, this buffer gave a pH of 4.

General Methods.—Methods for the determination of minimal lethal dose (M.L.D.) of tetanal toxin have been reported elsewhere (11). It has been found that strict adherence to these methods gives results which are reproducible to within 10 per cent. The combining capacity (Lf) and the time required for optimal flocculation (Kf) were determined by methods previously described (11). In most of the current experiments, increasing amounts of toxin were added to a constant amount of antitoxin. All other conditions were maintained as previously described. Since the antitoxin was maintained constant, the Kf of different samples was comparable. The criteria of Moloney and Hennessy (12) were rigidly followed in order to establish the true zone of flocculation. Special flocculating serums (13) were also employed to minimize the occurrence of false zones.

The techniques employed here for electrophoretic analysis have been reported (14). Ultracentrifugal studies were carried out by Dr. Dan H. Moore, of Columbia University, employing techniques in use in his laboratory. Solubility determinations were carried out according to Northrop (15). Isoelectric points were determined by a modification of the method of Michaelis and Rona (16). Hydrogen ion determinations were made on the glass electrode after warming the sample to 25° and before the addition of methanol. After adjustment of the pH and the addition of methanol, the ionic strength was calculated from the valence of the ions present and from their concentration. Centrifugation was carried out in a refrigerated centrifuge (International, PR-1). Constant temperature baths were employed to maintain the desired temperature of the solutions during fractionation.

Immunological Considerations.—The classical studies of Ramon have established the validity of the flocculation test for the determination of the combining units of tetanal toxin and toxoid. However, the flocculation test does not distinguish between toxin and toxoid, both of which combine with antitoxin. Therefore, in studies on the purification of toxins, caution must be observed in interpreting data based solely on flocculation tests. The intermediate or final products may represent mixtures of varying proportions of toxin and toxoid.

The toxic properties of toxins are due to groups or configurations on the molecule distinct from those groups responsible for flocculation. The determination of the M.L.D. of the toxin offers the best proof that toxin has not been converted to toxoid during fractionation or processing. The ratio of M.L.D. to Lf per mg. of nitrogen or per ml. of sample also offers evidence of the integrity of the toxin molecule and distinguishes between spontaneous "toxoiding" and denaturation during handling. A decrease in this ratio indicates that toxin is being converted to toxoid. A loss of Lf units concomitant with a decrease in this ratio is due to denaturation with a resulting loss of toxic properties and combining capacity. Any increase in this ratio indicates that toxoid is being separated from the toxin. The exact nature of the conversion of toxin to toxoid is unknown. However, certain evidence presented below and reported elsewhere (17) suggests that tetanal toxoid is a dimer of tetanal toxin molecules

which have apparently interacted or condensed through their toxic groups. Toxin which has spontaneously converted to toxoid exhibits immunological and physicochemical properties which differ from those of toxin. Thus, the presentation of data on a "pure toxin" may be misleading unless it is definitely established that the toxin is free of toxoid or that the toxoid has properties identical in all respects with those of toxin.

The Kf of toxin has been followed here to ascertain the extent of early denaturation of either toxin or toxoid. An increase in the Kf of a fraction compared with the preceding fraction or with the parent toxin is considered a definite indication of damage to the molecule. Any decrease in the Kf points to the removal of degraded toxin or toxoid molecules. Of course, such separations are desirable during purification and should occur if the fractionation procedure itself does not lead to irreversible changes in the labile proteins. In the present studies, the importance of Kf has been fully recognized. Any fraction showing evidence of a prolonged Kf is not employed for further fractionation or characterization.

Criteria of Purity.—The identification of a protein as a single molecular species is difficult. The commonly accepted criteria are constancy of solubility, constancy of chemical composition, and homogeneity of all measurable physical properties. When the protein has measurable functional activity, a constant correlation must exist between the above properties and all the biological functions of the protein. Such proteins as toxins, toxoids, and antitoxins must act as single substances in the flocculation test. In so far as possible, the conditions employed for physical characterizations should be within the physiologic range. This should permit a greater degree of correlation between physicochemical data and biological function of the protein. On the basis of data presented in this report, it would appear necessary to apply the above mentioned criteria to freshly prepared proteins. Identical samples should be employed for the comparison of biological activity with chemical and physicochemical data, and these measurements should be made simultaneously.

Methods of Purification.—The purification of toxins and toxoids offers many difficulties. One of the major problems is the separation of minute amounts of the active principle from large volumes of culture media and water. The instability of toxins, as well as their great affinity for surfaces and adsorbing agents, offers additional difficulties. Furthermore, contamination of the toxin by microorganisms capable of elaborating other lethal toxins and enzymes may alter the true M.L.D. value of the toxin. Finally, several closely related proteins are present as contaminants. Thus, the separation of toxins and toxoids requires fractionation systems other than salt precipitation, adsorption, or acid precipitation. The method must be reproducible and transferable from small scale laboratory experiments to the large scale production routinely employed by industry.

Theoretical considerations of the precipitation by ethanol-water mixtures

have been reviewed by Edsall (18). Cohn (19) and associates have put these theories into practice in the fractionation of plasma proteins. As pointed out by these workers, the denaturing effect of alcohol is minimized at the low temperatures employed. Dialysis, which is a major objection to neutral salt precipitations, is unnecessary in the process. The influence of pH, ionic strength, and protein concentration in this system of fractionation has been described (18).

In studies on the purification of labile toxins and toxoids (9, 11, 20-24) methanol has been found preferable to ethanol as the organic precipitating agent. Under certain conditions ethanol alters toxins even at low temperatures. It has also been noted in this laboratory that methanol may be preferable to ethanol for the fractionation of other proteins, including certain of the plasma proteins. The inherent danger of denaturation by increased temperature is greatly minimized by the use of methanol. Certain proteins which are denatured by ethanol at -5° are quite stable in methanol even at 0° . Since the volatile methanol is removed from the proteins by freezing the mixture and then removing both the water and alcohol at reduced pressure, clinical objections to the use of methanol are minimized.

For the most part, the influence of pH, alcohol concentration, temperature, and protein concentration on the solubility of toxins and toxoids is similar to that described for plasma proteins. The precise balance of the charged condition of proteins (which is determined by the pH of the mixture) and the methanol concentration plays the major rôle in the separation of toxin from bacterial proteins and culture medium constituents. Protein concentration is adjusted to allow protective stabilization of the toxin molecules, by virtue of protein-protein interaction, and of their dipolar activities. The adjustment of five independent variables permits the attainment of a large number of experimental conditions for the separation of the desired proteins.

The solubility of the components of a protein system will vary as a function of pH at constant ionic strength and constant methanol concentration, or as a function of methanol concentration at constant pH and constant ionic strength. Other sets of experimental values can be obtained as functions of temperature, ionic strength, or protein concentration at constant methanol concentration or pH. Each variable is adjusted at any one step in order to obtain the highest yield and greatest purity accompanied by no deleterious changes in the toxin molecules.

The conditions for removal of impurities at any one step in fractionation are specific for that step. In the same manner, the conditions required for the separation of a particular toxin will not necessarily apply to another toxin. Such investigations involve the laborious task of physicochemical, analytical, and immunological control and do not offer an easy or simple application to the purification of all protein systems. However, the precision of the method which

conforms to the disciplines of physical chemistry, the ease in obtaining highly pure products once the exact method has been established, and the reproducibility of results make it the method of choice in the fractionation of functional proteins.

RESULTS

In the present study the first step required that the toxin be removed as a precipitate with a minimum accompaniment of cytoplasmic bacterial proteins, and of proteoses, peptones, and amino acids which are constituents of the culture medium. In subsequent steps, further removal of these impurities was accomplished. Finally, the nucleoproteins, mucoids, and carbohydrates were removed, and then crystallization of the toxin was achieved. As will be seen below, the toxin may be precipitated or may be maintained in solution during the removal of impurities depending upon the limits of solubility of the protein and of the impurities, the nature of the impurities, and the convenience of the experimental conditions. It should be stressed that the conditions indicated in the text and tables must be rigidly followed for reproducibility. Small deviations in pH or other variables which may seem insignificant can result in a great decrease in yield and marked changes in the purification factors. Temperature should be controlled rigidly during the process. A cooling coil immersed in the toxin during the addition of methanol or the immersion of the toxin in cold baths with the slow addition of alcohol will greatly aid in maintaining the temperature near the freezing point or at -5° . Weston dial thermometers (stainless steel) make excellent stirring rods in this process because the temperature can be easily followed during processing.

Separation of Fraction T-PI.—In a series of solubility studies, the conditions for the separation of the toxin from culture medium products were determined. The general fractionation method follows.

One volume of toxin was chilled to 1° and adjusted to the desired pH with ice-cold acetate buffer or, in some instances, with acetic acid. To this mixture the calculated amount of methanol (measured at -5° and chilled to -20°) was added slowly with constant stirring, care being taken to maintain the temperature at -5° or under. The mixture was maintained at -5° for 24 hours. At alcohol concentrations above 40 per cent, the temperature was maintained at -10° , while at alcohol concentrations of 40 per cent or under, the temperature was kept near the freezing point or at -5° . The precipitate was removed in a refrigerated centrifuge at the same temperature as that used during processing. The precipitate was then dissolved to the desired volume with 0.15 M sodium acetate of pH 6.8. The solution was clarified by centrifugation at 4000 R.P.M. for 20 minutes at 1° , and immediately assayed or stored at -25° until needed.

Pertinent data on the behavior of the toxin in mixtures consisting of culture media, methanol, and water under varying conditions of pH, ionic strength, and temperature are summarized in Table I. Analysis of these results discloses the following information. Tetanal toxin is quantitatively precipitated be-

tween pH 4.5 and 5.25 in 40 per cent methanol at ionic strength 0.09 and at -5° . The highest concentration per milligram of precipitated nitrogen is obtained between pH 5.0 and 5.25. Hydrogen ion concentrations greater than 5.0 lead to the increased precipitation of constituents other than toxin. Hydrogen ion concentrations less than pH 5.25 result in an increased solubility of the toxin. Increasing or decreasing the ionic strength under the above conditions does not affect appreciably the yield or purity. Elevation of the temperature to 0° in 40 per cent methanol generally results in denaturation of the

TABLE I

Precipitation of Tetanal Toxin from Culture Medium in Methanol-Water Mixtures Varying in pH at Ionic Strength of 0.09 and at a Temperature of -5°C .

Conditions		Lf per mg. N	Kf*	M.L.D. per mg. N $\times 10^6$	Yield of parent toxin
pH	Methanol				
	<i>per cent</i>		<i>min.</i>		<i>per cent</i>
4.5	40	226	30		100
4.5	25	378	25	5.0	70
4.8	40	634	18	9.3	100
4.8	25	487	20	7.1	75
5.0	40	812	12	10.3	100
5.0	25	506	15	7.4	75
5.15	40	862	10	10.1	100
5.15	25	516	15	7.2	75
5.25	40	883	10	10.4	100
5.4	40	872	10	8.7	85
6.0	40	207	30	4.1	35
6.6	40	183	30		20
Parent toxin.....		6.9	30	0.068	

* Tested at 50 Lf units.

toxin.² Alcohol concentrations less than 40 per cent generally result in increased solubility of the toxin. Methanol concentrations greater than 40 per cent precipitate inert culture medium products and bacterial proteins. There is no evidence that hydrogen ion concentrations between 4 and 7 under the present conditions impair the toxin, since the Kf is often shorter than that of the parent toxin over the wide pH range. Pigment is precipitated with the toxin under pH 5.4.

The above information indicates that separation of the toxin from the crude toxic filtrate occurs optimally at pH 5.0 to 5.25 in 40 per cent methanol at -5° . The charged condition of the toxin has a greater influence on its solubility than the ionic strength of the mixture. The separation of bacterial toxins

² Such toxins show either an absence or marked retardation of flocculation.

differs in this respect from the separation of the plasma proteins in ethanol-water mixtures, which depends largely on precise salt concentrations (19). These results compare favorably with those reported for the purification of tetanal toxin produced in veal infusion medium (11). It should be noted that a single precipitation quantitatively separates toxin containing more than 800 Lf per mg. of nitrogen. Assuming that pure toxin has between 3300 and 3600 Lf per mg. of nitrogen, this material is over 20 per cent pure. This precipitate is hereafter referred to as T-PI. The ratio of M.L.D. to Lf units per mg. N is about 1.2×10^4 and is slightly greater than that of the crude toxin.

TABLE II

Precipitation of Tetanal Toxin from Fraction T-PI in Methanol-Water Mixtures Varying in pH, Ionic Strength, and Temperature

Conditions				Lf per mg. N	Kf*	M.L.D. per mg. N $\times 10^4$	Yield from T-PI
pH	Methanol	Ionic strength	Temperature				
	per cent		°C.		min.		per cent
3.5	25	0.057	-5	283	120		
3.5	10	0.068	-3	212	120		
4.0	25	0.057	-5	341	20	1.5	60
4.0	10	0.068	-3	364	20	1.5	15
4.5	20	0.06	-5	812	15	9.3	100
4.5	10	0.068	-3	974	15	12.6	83
5.0	20	0.06	-5	1113	12	18.3	100
5.0	10	0.068	-3	1282	12	22.0	100
5.5	25	0.037	-5	1150	12	20.6	100
5.5	15	0.024	-3	1390	12	24.2	100
6.0	25	0.057	-5	741	20	13.6	36
6.0	15	0.064	-3	1122	15	20.6	22

* Tested at 50 Lf units.

Solubility of Toxic Principle in Fraction T-PI.—Several large lots of crude tetanal toxin were processed to fraction T-PI. The initial separation of tetanal toxin from culture medium, conducted at pH 5.15 ± 0.05 in 40 per cent methanol at -5° , has been found to be reproducible for all crude toxins studied here. Fraction T-PI was dissolved to one-tenth of the parent toxin volume in 0.15 M sodium acetate. The behavior of this fraction in methanol-water mixtures was then observed over a wide range of experimental conditions. The results of these studies are summarized in Table II.

The toxin in fraction T-PI is quantitatively precipitated between pH 4.5 and 5.5 under appropriate conditions. It is insoluble in 20 per cent methanol at pH 4.5, in 10 per cent methanol at pH 5.0, and in 15 per cent methanol at pH 5.5. The removal of culture medium substances and bacterial impurities reduces the amount of methanol required to precipitate the toxin. It will be noted from the prolonged Kf that pH 3.5 apparently alters the toxin molecule

or precipitates degraded toxin or toxoid molecules. Hydrogen ion concentrations less than pH 5.5 result in increased solubility of the toxic principle under the observed conditions. The ionic strength apparently plays little or no rôle in the precipitation of toxin in this step. In view of the above results, the toxic principle is reprecipitated at either pH 5.5 in 15 per cent methanol or at pH 5.0 in 10 per cent methanol, at ionic strength between 0.025 and 0.075, and at a temperature of -3° to -5° . These fractions have an Lf content of about 1300 per mg. of N and about 20 million M.L.D. per mg. of N. The ratio of M.L.D. to

TABLE III

Solubility of Tetanal Toxin from Fraction T-P-II in Methanol-Water Mixtures and Water Varying in pH, Ionic Strength, and Temperature

Conditions				Lf per mg. N	Kf*	M.L.D. per mg. N $\times 10^3$	Yield from T-P-II
pH	Methanol	Ionic strength	Temperature				
	<i>per cent</i>		$^{\circ}\text{C.}$		<i>min.</i>		<i>per cent</i>
3.5	7.5	0.075	-5		60		90
3.5	5.0	0.075	0		60		90
3.5	0.0	0.075	0		60		90
4.0	10.0	0.075	-3	1712	5		80
4.0	10.0	0.025	-3	1483	5		60
4.0	7.5	0.075	-3	2080	5	42.1	92
4.0	7.5	0.05	-3	1562	5	31.9	86
4.0	7.5	0.025	-3	1100	5	27.8	60
4.0	7.5	0.15	-3	1538	15		92
4.0	5.0	0.075	0	1743	10		86
4.0	0.0	0.15	0	1320	15		92
4.0	0.0	0.075	0	1612	15		92
4.0	0.0	0.025	0	1612	15		86

* Tested at 50 Lf units.

Lf units per mg. of N is about 1.8×10^4 , which is considerably greater than the ratio in T-PI. This fraction is designated T-P-II.

Solubility of Toxic Principle in Fraction T-P-II.—Fraction T-P-II was dissolved to one-twentieth of the parent toxin volume in 0.075 M sodium acetate. Observations over a wide range of experimental conditions revealed that the separation of the toxic principle as a precipitate from the contaminating materials did not materially improve purification. Experiments were then designed to remove the bacterial proteins as a precipitate while leaving the toxin in solution. The results of an experiment with one large batch of T-P-II are given in Table III. All determinations given in this table were performed on the supernatants after the removal of the precipitated material.

It will be seen that most of the tetanal toxin remains in solution between pH 3.5 and 4.0 under appropriate conditions. Close analysis of the data reveals that at pH 3.5, with methanol concentrations of 7.5 per cent or lower, only a

slight precipitation of the toxin occurred. The K_f of the soluble toxin, however, is markedly prolonged indicating damage to the toxic principle. At pH 4.0 in either aqueous solution or in methanol up to 10 per cent, a large part of the toxic principle remains soluble with a sharp decrease in the K_f , indicating the removal of degraded toxin or toxoid molecules. It will also be observed that in the absence of methanol at this pH, the degree of purity is lower. For this batch of toxin, optimal separation of the toxin from bacterial protein occurs at pH 4.0, methanol concentration 7.5 per cent, ionic strength 0.075, at a temperature of -3° . Increasing the alcohol concentration at this pH results in a decreased yield of toxin in the supernatant, and decreasing the alcohol at this pH leads to incomplete removal of the impurities. The ionic strength at this step is of great importance. Whereas an ionic strength of 0.075 yields a toxic solution containing 2000 Lf per mg. of N, decreasing the ionic strength to 0.05 results in a product 25 per cent less pure. Further decrease of the ionic strength to 0.025 leads to a precipitation of toxin, and the supernatant has a purity of only 1100 Lf per mg. N. It is interesting that the removal of bacterial proteins under appropriate conditions markedly decreases the K_f of the resulting purified toxin.

The step which has just been described is of utmost importance in the purification of tetanal toxin. It has been noted that various lots of T-P_{II} behave differently at this step. It is, therefore, necessary to ascertain on an aliquot of T-P_{II} the optimal conditions for the separation of the bacterial proteins from the toxin. While this is a laborious procedure, it is imperative in order to obtain unaltered and highly purified toxin. For the adequate removal of the inert precipitate from T-P_{II} from twelve different batches of toxin, it has been observed that the conditions at this step vary from pH 3.8 to 4.2, from methanol concentrations of 5 per cent to 12 per cent, and from 0.05 to 0.15 ionic strength at temperatures between 0° and -5° . These fractions are designated T-S_{III}. The ratio of M.L.D. to Lf per mg. of N for these fractions is about 2×10^4 .

Solubility of Toxic Principle in Fraction T-S_{III}.—Fraction T-S_{III} was maintained at its freezing point and studies were carried out to remove other contaminants which may have remained in solution during separation of fraction T-S_{III}. It is still desirable to maintain the toxic principle in solution at this step. It was noted that the removal of nucleoproteins and other materials with low solubility at pH 4.0 allowed the addition of greater concentrations of methanol at this pH with little or no precipitation of the toxin. The results of these studies are given in Table IV. 90 per cent of the toxin remains soluble at pH 4.0 when the alcohol concentration is increased to 30 per cent. Increasing the pH leads to the precipitation of the toxic principle. On the basis of these studies, fraction T-S_{III} was adjusted to pH 4.0 and the alcohol concentration increased to 30 per cent. After several hours a flocculent precipitate settled out. The material was allowed to stand for 24 hours, and the supernatant was

collected by centrifugation at -5° and designated fraction T-SIV. This solution generally contains approximately 2500 Lf per mg. N.

Precipitation of Tetanal Toxin from Fraction T-SIV.—Various experimental conditions have been employed for the separation of tetanal toxin as a precipitate from fraction T-SIV. The general procedures employed for the separation of fraction T-II were followed. At this step, high ionic strengths should be avoided. It was observed that the precipitation of tetanal toxin at this point at ionic strengths greater than 0.025 resulted in irreversible changes in the toxin molecule. Furthermore, hydrogen ion concentrations less than pH 6.0 should also be avoided in order to retard the spontaneous conversion of tetanal toxin

TABLE IV

Solubility of Tetanal Toxin from Fraction T-SIII in Methanol-Water Mixtures Varying in pH

pH*	Methanol	Lf per mg. N†	Yield from T-SIII
	<i>per cent</i>		<i>per cent</i>
4.0	40	2800	60
4.0	30	2520	90
4.0	20	2118	100
4.2	25	2314	70
4.2	15	1982	80
4.5	25		40
4.5	15		40
5.0	25		10
5.0	15		10
5.5	25		
5.5	15		

* pH of T-SIII diluted with two parts of water.

† Supernatants tested.

to a flocculating atoxic protein. The most suitable conditions encountered so far for the precipitation of the toxin at this step are pH 5.4, 10 per cent methanol, ionic strength 0.02 at a temperature of -5° . A large quantity of sodium hydroxide is necessary to raise the pH of fraction T-SIV to 5.4. This, of course, increases the ionic strength of the solution. In order to avoid local excess of salt and hydroxyl ions, the alkali should be added in a dilute form in a methanol-water mixture to attain the above mentioned conditions. This addition should be made slowly with constant stirring. The material insoluble under these conditions is almost pure tetanal toxin. At times, some crystallization of the toxin occurs at this step. The precipitate was collected by centrifugation at -5° and was dissolved in 0.075 M sodium acetate to an Lf concentration of about 12,000 per ml. This fraction is designated T-PV and usually contains approximately 3000 Lf and 50 million M.L.D. per mg. of N.

Precipitation of Tetanal Toxin from Fraction T-PV.—Fraction T-PV, which

is almost pure tetanal toxin, was subjected to varying experimental conditions in order to remove traces of impurities prior to the crystallization of the toxin. It was found that reprecipitation of this material at pH 4.9 and ionic strength of 0.01 at 0° in the absence of methanol yielded the purest product. Therefore, fraction T-PV was diluted with ice-cold distilled water to an ionic strength of 0.01, the pH was adjusted to 4.9, and the material was allowed to stand for 24 hours at 0°. This precipitate was generally amorphous; however, crystals were often observed at this step. This fraction was designated T-PVI and was dissolved in 0.05 M sodium acetate to a concentration of about 12,000 Lf per ml. This solution contains about 3200 Lf and 60 million M.L.D. per mg. of N.

Crystallization of Tetanal Toxin.—The crystallization of tetanal toxin from fraction T-PVI is dependent on the purity of this fraction. Traces of nucleoprotein will interfere with crystallization. Furthermore, if even partial spontaneous detoxification of the toxin has occurred during processing, crystallization is retarded. It is obvious that if the special precautions indicated in the above procedures are not followed to avoid denaturation of the toxin, crystallization will not be accomplished.

A number of experimental conditions were employed to achieve crystallization. Tetanal toxin readily forms an amorphous precipitate in aqueous solutions between pH 4.5 and 5.4 resembling a "euglobulin" in the classical terminology. However, in very dilute solutions, under these conditions of pH and in the presence of optimal concentrations of methanol, the toxin will crystallize out of solution. This procedure was employed early in the experimental work. While this procedure yields adequate quantities of crystals, it involves the handling of large volumes of material and is time-consuming. Attempts have been made to establish more practical conditions for the crystallization of tetanal toxin. Crystallization of the toxin does not occur readily at a pH acid to its isoelectric point. However, if the hydrogen ion concentration is sufficiently low (between pH 5.8 and 6.2), the addition of alcohol up to 20 per cent concentration does not produce an amorphous precipitate. Fraction T-PVI was adjusted to a 1 per cent protein solution at pH 6.0, methanol concentration of 20 per cent, ionic strength of 0.02, and a temperature of -5°. Crystallization of the toxin occurred within a few days, and on standing for several weeks, a good yield of crystals was obtained. Attempts to obtain crystals at hydrogen ion concentrations less than pH 6.0 were not highly successful, since at these hydrogen ion concentrations tetanal toxin is readily detoxified. Hydrogen ion concentrations greater than pH 6.0 resulted in a precipitate of both crystalline and amorphous material. A summary of the degree of purity and yield of tetanal toxin at each step is given in Table V. The conditions of pH and methanol concentration employed at each step are shown graphically in Fig. 1.

The crystals obtained can be redissolved in sodium acetate and recrystallized under the above stated conditions. The final product can be dissolved either

in 0.15 M sodium chloride at pH 5.5 or, for greater stability, in 0.3 M glycine at pH 5.8: This material can be stored at -25° , dried from the frozen state, or

TABLE V
Degree of Purity and Yield of Tetanal Toxin at Various Fractionation Steps
(Average Values)

Fraction	Lf per mg. N	M.L.D. per mg. $N \times 10^5$	Yield
Parent	6-10	0.06-0.1	<i>per cent</i>
T-PI	800-1000	10-12	95-100
T-PII	1200-1400	18-24	90-100
T-SIII	1800-2400	36-45	70-80
T-SIV	2200-2600		70-80
T-PV	2800-3200	40-50	60-80
T-PVI	3000-3400	50-60	50-70
T-CVII	3300-3600	60-70	10-40
T-CVII-4	3300-3600	60-70	5-35

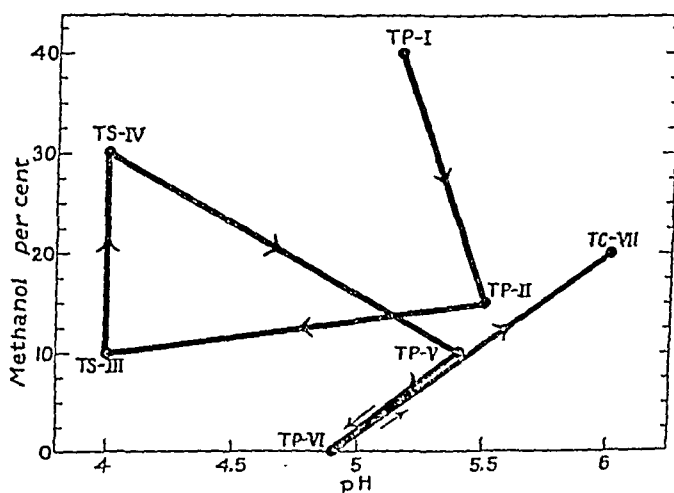


FIG. 1. A schematic diagram of the conditions of pH and methanol concentration at different steps of the purification of tetanal toxin. P, toxin precipitated under described conditions; S, toxin soluble under described conditions; C, toxin crystallized under described conditions.

maintained at 0° in the presence of 0.3 M glycine. A photograph of the crystals has been published previously (9).

Properties of Crystalline Tetanal Toxin

Crystalline tetanal toxin in a 1 per cent solution is pale yellow. It is very stable in the presence of glycine between pH 5.0 and 6.0. It is relatively stable

in the presence of neutral salt at this pH. However, hydrogen ion concentrations less than 6.0 lead to the spontaneous conversion of the toxin to a flocculating atoxic protein.

TABLE VI
Chemical Composition of Crystalline Tetanal Toxin

Nitrogen, <i>per cent</i>	15.7
Phosphorus, <i>per cent</i>	0.065
Sulfur, <i>per cent</i>	1.04
Carbohydrate.....	—

TABLE VII
Properties of Crystalline Tetanal Toxin

Mobility in veronal buffer, pH 8.6, 0.1 ionic strength.....	2.8×10^{-5}
Sedimentation constant, s_{20}^{w}	4.5
Optical rotation, $[\alpha]_D^{25}$, degrees.....	-63
Isoelectric point.....	5.1 ± 0.1
Lf per mg. N.....	3600
Kf ₅₀ , min.....	10
M.L.D. per mg. N.....	6.6×10^7

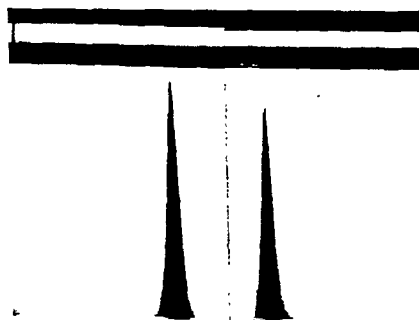


FIG. 2. Electrophoretic pattern of crystalline tetanal toxin. Time = 18,000 seconds.

Chemical composition of the toxin is summarized in Table VI. While the crystalline toxin gives the usual protein reactions and contains 1 per cent sulfur and a trace of phosphorus, it is entirely lacking in carbohydrate. Amino acid analyses of the crystalline protein are being conducted by Dr. Max S. Dunn and will be reported later.

Table VII summarizes the physicochemical character of freshly dissolved crystalline tetanal toxin. The toxin is electrophoretically homogeneous (Fig. 2) with a mobility of 2.8×10^{-5} in veronal buffer of 0.1 ionic strength at pH 8.6. The optical rotation of -63° differs from the optical rotation of -45° for diphtherial toxoid (21).

The isoelectric point was estimated from the minimum solubility of both total N and the combining activity of the crystalline toxin. The toxin was adjusted to different hydrogen ion concentrations with acetic acid at 0.2 pH intervals in distilled water at constant volume and at 1°. The results of this experiment

TABLE VIII
Solubility of Crystalline Tetanal Toxin in Distilled Water as Function of pH

pH*	Li precipitated	Protein N precipitated
	<i>per cent</i>	<i>per cent</i>
4.3	0	0
4.5	30	30
5.0	90	90
5.2	90	90
5.5	40	40
6.0	20	20

* Determined on supernatants after equilibration with the precipitates for 18 hours at 1°

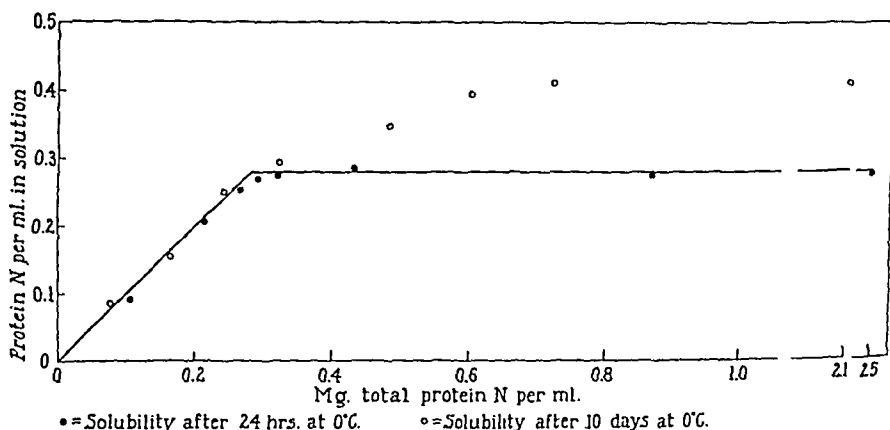


FIG. 3. Solubility curves of crystalline tetanal toxin. The protein nitrogen per milliliter in solution is plotted against the total protein nitrogen concentration. The solid line is the theoretical curve for a solid phase of one component.

are given in Table VIII. It will be noted that, judged both by N and by combining capacity, the toxin has a minimum solubility at $\text{pH } 5.1 \pm 0.1$.

As pointed out above, crystalline tetanal toxin, on standing at 0° rapidly loses toxicity with no loss in its ability to flocculate with tetanal antitoxin. This phenomenon complicated the determination of the exact sedimentation constant and true constant solubility of the crystalline protein. Inspection of Fig. 3 reveals that the solubility of the freshly prepared crystalline protein is almost constant in 1.4 M ammonium sulfate at pH 7.0 and acts as though it were essentially a solid phase of one component. However, on standing for 10 days at

0°, at least 50 per cent of the material shows a change in solubility and the solution appears to contain two molecular species. This change is accompanied by a loss of about 75 per cent of the toxicity of the solution while the flocculating capacity remains unaltered. This would indicate that the solubility of freshly

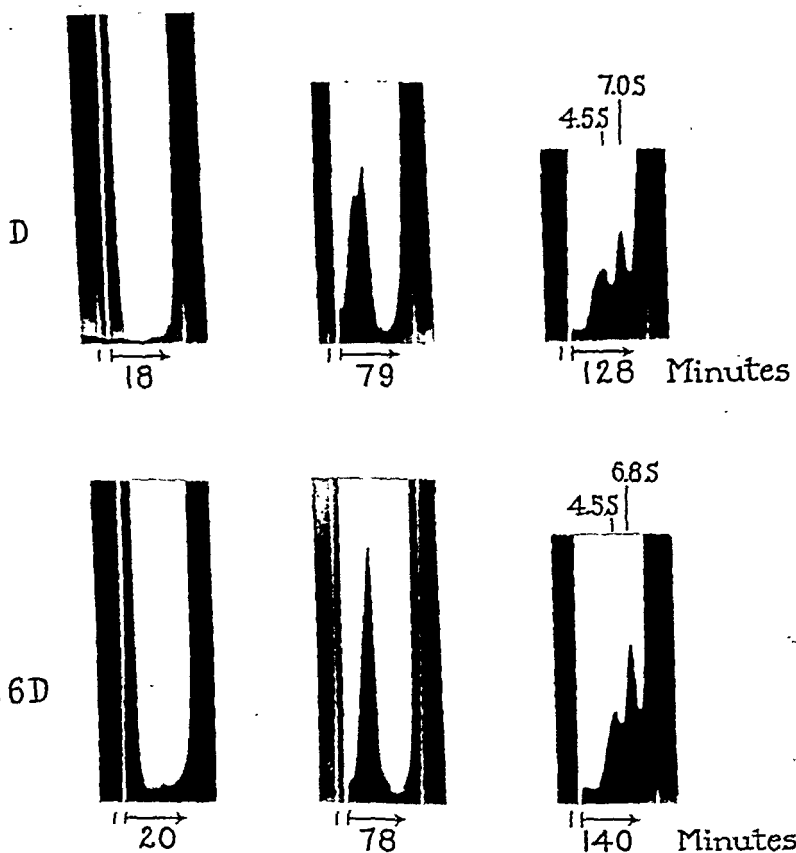


FIG. 4. Sedimentation patterns of crystalline tetanal toxin after standing for 10 days at 0°. *D*, once crystallized toxin. *.6D*, four times recrystallized toxin.

prepared crystalline toxin is constant and that the changes observed on standing are caused by the condensation of the pure toxin. This hypothesis was substantiated by the results of ultracentrifugal studies (17). The unavailability of an ultracentrifuge in this laboratory at present made it impossible to study freshly prepared crystalline toxin. Samples were forwarded to Dr. Dan H. Moore, Columbia University, who conducted the ultracentrifugal studies. It will be noted that 55 to 60 per cent of the molecules had a sedimentation constant of 7.0 and the remaining molecules sedimented at 4.5 S (Fig. 4). Immu-

nological analysis of these fractions revealed that the toxin was associated with the molecules sedimenting at 4.5 S. Both fractions flocculated with antitoxin. These results suggest that crystalline tetanal toxin is spontaneously converted to a flocculating atoxic dimer. Work will be carried out to determine the mechanism of this reaction. In reviewing the work of other investigators on the purification of toxins, little or no reference is made to the possibility that their final toxin preparations may have contained toxoid. The spontaneous conversion of crude toxin to toxoid (natural toxoiding) is well known. It seems highly improbable that the usual methods employed for the fractionation of toxins involving dialysis and extraphysiologic conditions of pH, salt concentration, and temperature would not have caused some conversion of the toxin to toxoid. That such a conversion might have taken place is indicated by the absence of constant solubility data for purified toxins. The recognition of the possibility that toxin may easily be converted spontaneously to a toxoid should lead to a better understanding and to more exact physicochemical data on these substances. On the basis of the present studies it appears that tetanal toxin has a sedimentation constant of 4.5 Svedberg units.³

Crystalline toxin is not precipitated by an anti-*Clostridium tetani* rabbit serum. The toxin is almost immediately detoxified by very small amounts of formalin (0.01 to 0.001 per cent). This detoxified material is highly antigenic and can be used for immunization. However, the inherent technical hazards to personnel during the processing of the toxin makes it advisable to employ crude toxoid for the parent material when a purified toxoid is desired. The conditions described in the text for the purification of tetanal toxin are the same for the purification of tetanal toxoid with the following exception: in the removal of T-PI the pH should be adjusted to 4.85 ± 0.05 .

SUMMARY

Methods for the purification and crystallization of tetanal toxin are described. The methods consist of the multiphase fractionation system involving methanol as the organic precipitating agent under controlled conditions of pH, ionic strength, protein concentration, and temperature.

Crystalline tetanal toxin has an electrophoretic mobility of 2.8×10^{-5} in veronal buffer of 0.1 ionic strength at pH 8.6. The solubility of freshly prepared toxin is essentially constant. The isoelectric point is 5.1 ± 0.1 . The crystalline toxin contains 1 per cent sulfur, traces of phosphorus, and gives the usual protein reactions. It does not contain carbohydrate.

³ Since this paper has gone to press, samples of crystalline tetanal toxin have been stored at 0° in the presence of homogeneous human albumin (4.5 S). It has been noted that no loss either in the toxicity or in the combining capacity of the toxin occurred after standing for 20 days. Ultracentrifugal analysis of this mixture revealed that the sample was homogeneous with a sedimentation constant of about 4.5. This substantiates the view that tetanal toxin has a sedimentation constant of 4.5.

The crystalline toxin does not precipitate anti-*Clostridium tetani* rabbit serum. The final product contains between 3400 and 3600 Lf and about 6.6×10^7 M.L.D. per mg. N.

Crystalline tetanal toxin is spontaneously converted to a flocculating atoxic dimer upon standing at 0°. This change is accompanied by the appearance of another molecular species as judged by constant solubility tests. Ultracentrifugal analysis of these fractions reveals that tetanal toxin has a sedimentation constant of 4.5 Svedberg units while the atoxic flocculating dimer sediments at 7 S.

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ISOLATION OF MUMPS VIRUS FROM HUMAN BEINGS WITH INDUCED APPARENT OR INAPPARENT INFECTIONS*

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Many deductions concerning the spread of mumps virus were made from clinical and epidemiologic observations before confirmation by laboratory tests was possible. The evidence presented by various authors in regard to this subject has been critically reviewed by Gordon (1). The following conclusions appeared justified on epidemiologic grounds: (a) The average incubation period of typical mumps was shown to be 18 days. The disease was found to become communicable about 48 hours prior to the onset of glandular enlargement and the period of communicability to last for several days following the appearance of signs of illness. (b) Cases of unusual manifestations of mumps, such as primary meningoencephalitis or primary orchitis in the absence of recognized involvement of the salivary glands, could be properly classified only if they occurred in institutions or families where typical mumps was prevalent. It was shown, on occasion, that these patients were able to transmit mumps to contacts, possibly because of the presence of virus in their saliva at some stage of the infection. (c) Inapparent infections with mumps virus were thought to be frequent, since in spite of the prevalence of the disease only about 60 per cent of the adult population of the United States had suffered clinical attacks. It was also inferred that such inapparent cases of infection might spread the virus to susceptible individuals, thus maintaining the chain of infection in an epidemic.

With the development of laboratory tests for the diagnosis of infections with mumps virus (2, 3) and of technics for the isolation of the agent in chick embryos (4, 5), it has become possible to confirm many of the earlier observations by more direct proof. Thus, the existence of inapparent infections and their relative frequency has been demonstrated by application of complement fixation tests (2, 6, 7). By the same means, the diagnosis of unusual manifestations of mumps has become a routine matter (8, 3). Virus has been isolated from the saliva of cases of parotitis as late as 6 days after onset of disease (9). It has also been demonstrated in the spinal fluid of patients with meningoencephalitis without salivary gland involvement (10, 11).

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The present paper is concerned with the isolation of mumps virus from the saliva of human beings at various stages of infection. It was considered difficult to approach these problems under epidemic conditions since frequently the time and intensity of exposure would not be accurately known, whereas the study of cases experimentally exposed to mumps virus offered the advantage of controlling both these factors. The results obtained under such experimental conditions afford evidence that the period of infectivity of cases with involvement of the salivary glands may begin as early as 6 days prior to onset of recognizable glandular swelling, and may last for more than one week. It will also be shown that a patient with orchitis without salivary gland involvement harbored mumps virus in his saliva at some time prior to the development of symptoms. Finally, it will be demonstrated that cases of inapparent infection may excrete virus in saliva for considerable periods of time and that they may be responsible for spreading the disease in an epidemic.

Methods and Materials

Selection of Susceptible Subjects.—Institutionalized children in good physical condition and without known histories of mumps were bled and their sera tested for antibodies against mumps complement fixation antigens. Those children whose sera failed to react with the soluble and the virus antigens (12) were considered susceptible to mumps and, therefore, chosen for these experiments with the permission of parents and guardians.

Virus.—Two strains of mumps virus (F and B) were used. They had been isolated from the saliva of cases of parotitis by amniotic inoculation of chick embryos (13). Amniotic fluid of the fifth passage was quick frozen and stored in glass-sealed ampules in a dry-ice cabinet until used. The preparations of both strains contained 10^7 50 per cent infectivity doses for chick embryos per ml. and 5000 (F) and 7500 (B) hemagglutinating units.

Isolation of Virus from Saliva.—Saliva or mouth washings were obtained in the first experiment, depending on the ability of the children to furnish saliva. In the second experiment saliva was mechanically aspirated by means of a device consisting of a thin bent copper tube leading into a glass trap which was in turn connected with an electrically driven suction pump. Individual equipment was used for each child. The specimens were immediately transferred into ampules which were sealed off in an oxygen flame. After quick freezing the ampules were transported to the laboratory packed in dry-ice. They were stored at $-70^{\circ}\text{C}.$ until inoculation of chick embryos was possible.

Before injection the specimens were thawed and centrifuged at 2000 R.P.M. for 10 minutes. The supernatant fluids were incubated at $37^{\circ}\text{C}.$ for 1 hour with penicillin and streptomycin, using 1000 units of each per ml. The specimens were injected in 0.2 ml. amounts into the amniotic cavity of ten 8-day-old chick embryos, under direct observation as described previously (12). After further incubation for 5 days at $36-37^{\circ}\text{C}.$ the eggs were chilled for 1 to 3 hours at $4^{\circ}\text{C}.$ and the amniotic fluids were harvested aseptically. These were tested individually on slides for their ability to agglutinate chicken red cells. Further transfers were made by injecting undiluted amniotic fluids into new groups of eggs. A specimen of saliva was considered negative if three amniotic passages failed to indicate the presence of virus.

For the identification of the isolated strains of virus pools of amniotic fluids of the respective groups of eggs were used as antigens for complement fixation tests with known acute and convalescent sera of patients with mumps. In all cases tested the agent isolated was identified as mumps virus.

Serologic Tests.—Blood was drawn from all individuals at intervals to study the development of complement-fixing antibodies. The technics and the antigens employed have been fully described in previous communications (3, 12).

EXPERIMENTAL

Two experiments were conducted with a total of 15 children. The experiments differed in regard to the amount of virus to which the subjects were exposed and the method by which it was applied. In all other respects the two tests were essentially alike. Before exposure to the virus the children were isolated in a hospital ward where they remained for the period of observation, under the care of trained nurses. Their temperatures were recorded twice daily, and 4 times daily in cases in which fever was observed. Beginning 2 weeks after exposure the children were examined daily for signs and symptoms of disease by one of the authors, except for a few occasions when adverse weather conditions rendered the institution inaccessible.

Experiment 1 (November, 1947).—Seven children were exposed to active mumps virus which was deposited by means of a coarse spray on the mucous membrane of the oral cavity, particularly close to the orifices of Stensen's duct. Four of the children received 2.0 ml. of amniotic fluid containing strain F, and three children were infected with the same amount of strain B. The amount of virus sprayed for each child corresponded to 2×10^7 ID₅₀ for chick embryos in the case of both strains.

Experiment 2 (January, 1948).—In the second experiment, eight subjects were exposed to finely dispersed virus, by means of an atomizer,¹ operated by compressed air. Thus the children inhaled small droplets and droplet nuclei through their mouths. A mixture of equal parts of strains F and B was employed. Four of the subjects were exposed to a spray of 1.0 ml. of a 1:100 dilution of infected amniotic fluid, containing 10^8 ID₅₀ for chick embryos, and the remaining children inhaled 1.0 ml. of undiluted fluid containing 10^7 ID₅₀.

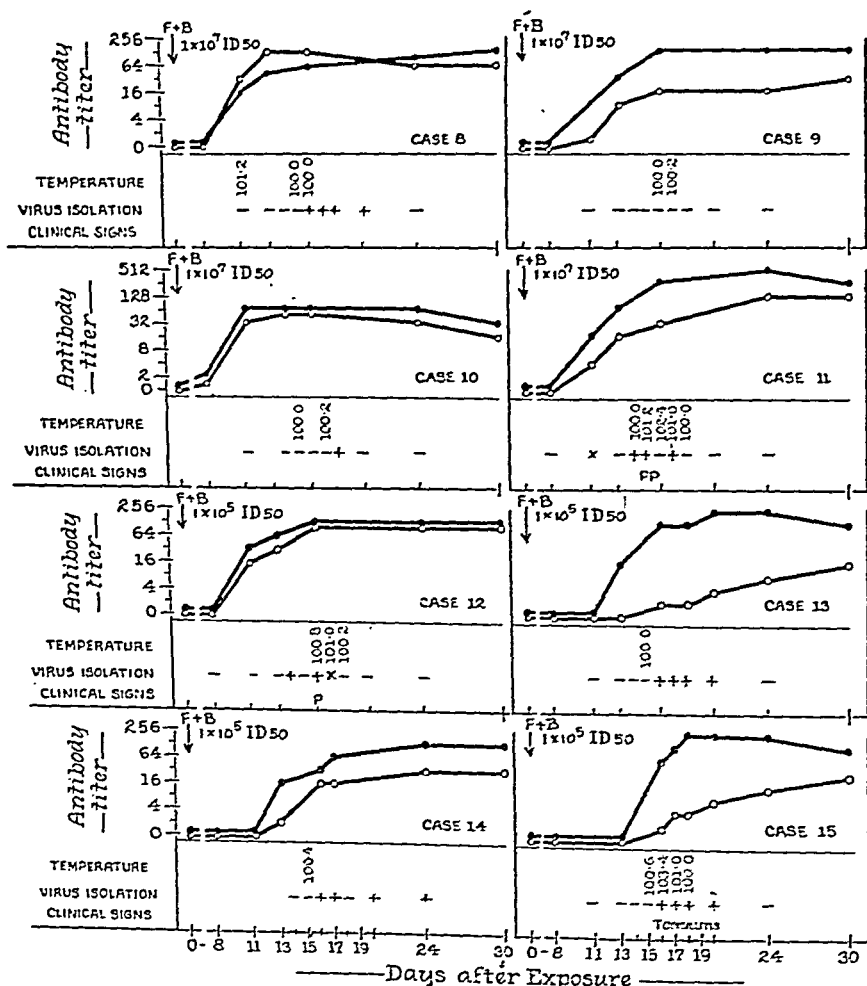
Since the clinical observations and the laboratory findings of the two experiments were similar, they will be discussed together. A summary of all the data is presented in Figs. 1 and 2.

Clinical Observations.—As can be seen from the figures, four of the fifteen children came down with a clinically well defined parotitis (cases 2, 7, 11, and 12). Two additional cases (Nos. 1 and 5) showed signs of involvement of the submaxillary glands. Case 6 developed orchitis without parotitis. One case (No. 15) came down with tonsillitis and palpable lymph nodes 17 days after exposure to mumps virus. The purulent exudate and the high temperature suggested an intercurrent bacterial infection, which was promptly relieved by chemotherapy. The additional seven children (cases 3, 4, 8, 9, 10, 13, and 14) remained well during the period of observation except for minor febrile responses. The significance of these elevations of temperature is questionable.

The incubation periods fell into the range encountered in the epidemic disease. The first case of parotitis (No. 7) was observed on the 14th day after

¹ Kindly supplied by the Vaponefrin Company, Upper Darby, Pennsylvania.

Serologic Results.—Complement fixation tests were performed with sera taken from all children at intervals during the course of the experiments. All cases



The eight children who failed to exhibit signs of mumps showed antibody responses similar to those of the children who developed clinical illness. These cases must be classified, then, as inapparent infections. The rate of antibody formation against the V antigen was somewhat delayed in three of the children (cases 5, 13, and 15).

Isolation of Virus.—Series of specimens of saliva were thus available from (a) six patients with involvement of the salivary glands; (b) one patient with a manifestation of mumps without salivary gland involvement (orchitis); and (c) eight subjects who were classified as having inapparent infections according to their serological response. This group includes the patient with tonsillitis.

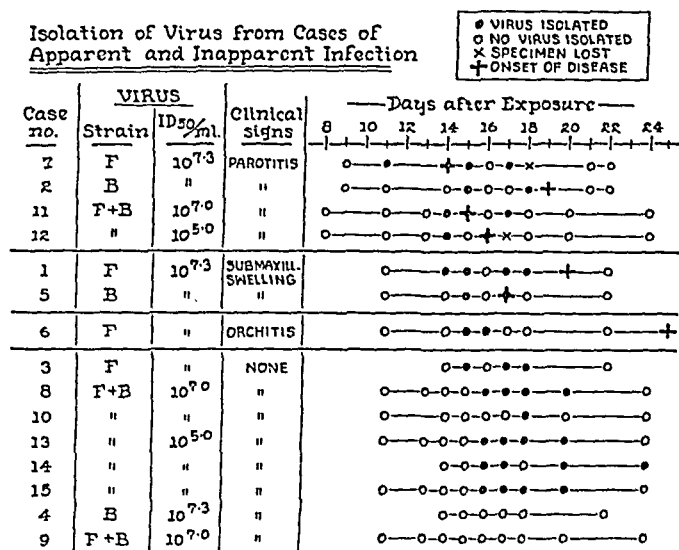


FIG. 3

Figs. 1 and 2 show the attempts and successful isolations obtained from each of the subjects in relation to clinical and serological findings. Fig. 3 summarizes the result of the isolation procedures, grouped according to the clinical signs of infection seen in the children. The positive findings require no comment. The negative data, on the other hand, do not necessarily indicate that no virus was excreted on the day the specimen was obtained. As can be seen, negative results were recorded in some instances in which the specimens of the preceding and following days were positive. It is possible that the technic of sampling of the saliva was inadequate or that the excretion of virus occurred intermittently. The collection of specimens was unfortunately less regular in the later days of the two experiments because of snow storms, which prevented traveling to the institution on several occasions. Attempts to isolate virus on the 1st and 2nd days after exposure failed. Later in the experimental period, as can be seen in Fig. 3, all children who showed clinical signs of parotitis or of

submaxillary swelling excreted virus for several days. In all these cases, virus was isolated 2 to 6 days prior to the appearance of swelling, beginning at the 11th to 15th day after spraying. Virus was also demonstrated in some of the samples of saliva taken after onset of the disease. Thus in case 7 virus was still present on the 4th day of illness.

Isolation of virus was not only successful in the cases just described but also from the saliva of the case of orchitis, who showed no involvement of the salivary glands. Virus was found on the 15th and 16th days after exposure to mumps virus, i.e., 10 and 9 days prior to the onset of orchitis. In addition, virus was isolated from the saliva of six of the eight children who experienced

TABLE I
Isolation of Virus from the Saliva of Representative Cases

Case No.	Passage No.	Time after exposure, days										Period of excretion
		11	13	14	15	16	17	1	20	22	24	
1	1	0/8*		0/9	3/5	0/8	3/6	6/6		0/8		At least 5
	2	0/5		0/8	5/6	0/6	6/7			0/7		
	3	0/8		8/8		0/6						
12	1	0/8	0/6	0/6	0/6	0/6	‡	0/3	0/6		0/5	3-4
	2	0/7	0/6	1/5	0/7	5/5		0/6	0/7		0/8	
	3	0/8	0/4	6/6	0/7			0/8	0/8		0/8	

Bold-faced type is used to emphasize the positive isolations.

* None of eight eggs showed positive hemagglutination on 5th day after inoculation. Two of the ten originally injected embryos died of non-specific causes during the incubation period.

‡ All embryos died on account of bacterial contamination.

inapparent infections (cases 3, 8, 10, 13, 14, and 15) at about the same time as from the clinical cases of mumps. As shown, virus was isolated from these children for periods of 1 to 9 days. Only children 4 and 9 failed to reveal virus in any of the available specimens.

These data show then that virus could be isolated from thirteen out of fifteen children exposed to mumps virus under experimental conditions. Table I gives the results of the isolation of virus in two of the children. As can be seen, some of the specimens of saliva yielded, on first passage in the majority of the injected eggs, sufficient concentrations of virus to cause hemagglutination. With other samples two, or in one instance even three, passages were required before positive results were obtained. A specimen which failed to yield hemagglutination by the third passage was considered free of virus. The allantoic fluids of these eggs never showed hemagglutinating properties. The

death rate in chick embryos inoculated with bacteriologically sterile saliva was frequently high. In other instances the antibiotic failed to inhibit bacterial multiplication and a few entire groups of eggs were lost because of such contamination.

DISCUSSION

It is obvious that no quantitative and statistically valid conclusions can be drawn from the data presented, since these were derived from only a small number of cases of induced infection with mumps virus. However, the data confirm experimentally some of the earlier conclusions deduced from epidemiologic observations (1). Thus, the incidence of inapparent infections in mumps has been estimated to be of the order of 30 to 40 per cent. Serological analysis of epidemics in recent years has confirmed this contention in that a high percentage of exposed subjects developed complement-fixing antibodies for mumps in the absence of clinical signs of infection (2, 6, 7). The experimental exposure leads to similar results. In the present series, eight of the fifteen exposed children must be classified as having had inapparent infections.

The period of communicability of the experimental cases showing involvement of the salivary glands began, according to the results of isolation of virus, on the 11th to the 15th day after exposure and extended to the 18th to 19th day. Thus, virus appeared in the saliva 2 to 6 days prior to the appearance of glandular swelling, and it was found as late as 4 days after onset of the clinical signs. Leymaster and Ward (9) obtained virus from one case of epidemic mumps on the 6th day of disease. It is possible that the experimental results are strongly influenced by the intensity of exposure. A smaller dose of virus ($<10^6$ ID₅₀) might conceivably delay the onset of viral excretion for a few days, as well as prolong the incubation period.

Most of the children who failed to show clinical signs of involvement of the salivary glands nevertheless excreted virus in their saliva at certain times after exposure. One of these developed orchitis at a later stage. It had been shown in the past on epidemiological grounds that such cases of unusual manifestations of mumps, including meningoencephalitis without parotitis, may spread the infections to susceptible individuals (14, 15).

The isolation of virus from the cases of inapparent infection was successful over a period of from 15 to 24 days after exposure. They became "transitory carriers" for from 1 to 9 days or longer. Unfortunately, no specimens of saliva were collected after the 24th day. However, there are strong indications that one of the children of the second experiment, classified as having inapparent infection, was still harboring the virus on the 30th day after exposure, when the group were released from the hospital and returned to their cottage. Between 15 and 18 days thereafter, four cases of parotitis occurred in that cottage in children not belonging to the group. Although there is no definite proof that

the virus was derived from one of the returning children, the possibility cannot be denied, because of the time relationships encountered. Whether the virus, if introduced by one of the children, was spread through the saliva, or whether it was present on contaminated clothing cannot be determined in this case. However, transmission by contaminated apparel has generally been disregarded as a source of infection (1).

It appears, then, that the virus used for experimental exposure closely resembled the epidemic agent and had not become attenuated to any large extent as a result of five passages in the chick embryo. The data, in addition, seem in good agreement with the earlier epidemiologic observations. However, certain differences were noted in the rate of production of antibodies in the experimentally exposed subjects in comparison with cases observed under epidemic conditions. In the natural disease antibodies to the soluble antigen may be present at the onset of swelling, or they rise very shortly thereafter; antibodies to the virus antigen usually appear in measurable quantities several days later, when antibodies to the soluble antigen have reached high levels. Only in the case of complications, such as orchitis, may high titers to both antigens be found at the time of their onset. On the other hand, in the experimental groups, distinct antibody levels for both antigens were found in the cases with involvement of the salivary glands, several days before clinical signs of the disease, with the exception of one patient who showed a low titer with the virus antigen at the time of the submaxillary swelling. The patient with orchitis had high antibody levels 10 days before he showed signs of illness, and subsequently a second rise in antibodies to both antigens was found. It is possible that the findings described are the result of the rather intensive exposure under experimental conditions since most of the children were sprayed with 10^7 ID₅₀ (for chick embryos). When the dose was decreased to 10^5 ID₅₀ the antibodies to the V antigen appeared at a slower rate in two of the three cases who were classified as inapparent infections (cases 13 and 15).

There does not seem to be a relationship between the development of complement-fixing antibodies and the excretion of virus in saliva in the cases described. With the exception of case 3, whose antibody levels were the lowest in the two groups, antibody titers to the soluble antigen were high and, in some cases antibodies to the virus antigen were measurable in large amounts when virus was isolated from the saliva. The relationship between complement-fixing and neutralizing antibodies has not been fully established. However, preliminary experiments with sera of some of the cases revealed that neutralizing antibodies were circulating in the blood stream at a time when virus was found in the saliva. It seems possible that after the virus has invaded the salivary glands it is no longer accessible to humoral antibodies unless they reach such high concentrations in the blood as to be reflected in the secretions. Such a situation would not be unlike that observed in the case of infections of man with influenza virus. These questions will be the subject of further studies.

SUMMARY

Exposure of fifteen children to mumps virus of fifth amniotic passage in chick embryos led to involvement of the salivary glands in six, orchitis in the absence of other manifestations of mumps in one, and to no signs of illness in eight. Attempts to isolate virus from the saliva of these individuals gave the following results:

1. All patients with involvement of the salivary glands excreted virus beginning on the 11th to 15th day after exposure, 2 to 6 days prior to onset of clinical signs of disease and extending up to the 4th day of illness.

2. The patient with primary orchitis without any recognized involvement of the salivary glands excreted virus for 2 days, beginning on the 15th day after exposure and 10 days prior to his illness.

3. Six of the eight children classified as having inapparent infections because of their serologic response in the absence of clinical signs of illness, began to excrete virus on the 15th to 16th day after exposure for from 1 to 9 or more days.

The epidemiologic significance of these data is discussed.

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STUDIES ON NEWCASTLE DISEASE VIRUS

I. AN EVALUATION OF THE METHOD OF TITRATION

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Electron microscope studies of purified preparations of the virus of Newcastle disease have shown that this virus has a filamentous to sperm-like form when dried from saline suspensions (1, 2). If, however, the virus is suspended in water and then dried from this suspension, it has a roughly spherical form, which corresponds to its appearance in the original allantoic fluid (3). An apparent conversion from the spherical form in water to the filamentous form has been brought about by the addition of saline to make a 0.07 to 0.15 M solution (3). The change in shape revealed by electron microscopy was brought about without any detectable change in activity of the suspension. Two questions must be answered before these observations can be assigned any basic significance. (a) Could the lack of a detectable change in activity be merely due to inadequacies in the methods of measuring activity? In other words, might we destroy 95 per cent of the virus by the procedure which changes its shape, and not detect this loss of active virus particles? (b) Does this change in shape on transfer to saline represent a change which occurs within the solution, or is it merely the result of drying from different solvents? Some experimental evidence against this latter possibility was previously mentioned (3), but more work needs to be done.

We attempt in this paper to evaluate the methods of determining activity (embryo infectivity) and conclude that the changes in shape of the virus cannot be accounted for on the basis of disintegration.

The determination of the 50 per cent mortality or infectivity end-point (4) by means of chick embryos has been in use for a number of years, but very few reports have been made on the accuracy of the method used. Indeed, there is no statistical study available which attempts to estimate the expected probable error with a given number of animals and a given titration end-point slope.

In a limited number of duplicate titrations of Eastern equine encephalomyelitis, a variation of as much as 0.6 log was found between two duplicate titrations (5), using five embryos in each of three dilutions. In a more extensive evaluation of the 50 per cent infectivity measurements for influenza virus in the embryo, Knight (6) found that the chances were 19 out of 20 that differences in end-points of 0.62 logarithmic units were significant.¹ This means that more than 75 per cent of the infectiousness

¹ Five embryos in each dilution.

of a preparation must be destroyed before the odds are as much as 20 to 1 that any decrease at all can be stated to have occurred. The accuracy is, of course, increased by increasing the number of embryos used.

The consistency of results obtained by Sigurdsson (7) when measuring the rate of multiplication of vesicular stomatitis virus in 10-day-old embryos would indicate a roughly similar accuracy for this virus when titered on 7-day-old embryos. Similar consistent results have been obtained in measuring the concentration of equine encephalomyelitis after 23 hours' inoculation (5).

It is, however, at present impossible to predict the accuracy of the method for a new virus infection since the sharpness of the end-point in itself would influence the accuracy of the determination. Quantitative determinations of the sharpness of the end-point, or end-point titration curves are available for only a few animal viruses (8-11) and those not on the embryo.

In a later paper in this series it is shown that infection and death of the embryo are produced by a very few particles of Newcastle virus (12). By use of the theory of random distribution of particles as outlined in the Poisson theory, it is possible to draw a theoretical curve for the expected number of infectious units needed to produce infection. This may be illustrated by two extremes. If one unit is capable of producing infection and a solution containing just enough material to produce infection is diluted $\frac{1}{10}$, then the inoculation to ten times as many embryos with this dilution produces the same total number of infections. If, however, it is necessary to add 1000 infectious units to an embryo to initiate an infection, and a drop of a given suspension contains this much, an equal portion of $\frac{1}{10}$ dilution would rarely have sufficient particles in it to initiate infection. Therefore, the next tenfold dilution would almost always be negative.

These considerations are raised here because they have direct bearing on the accuracy of the method. Fig. 1 shows that the points obtained with small dilutions of the virus best fit a one particle (infectious unit) curve. This does not prove that infection is produced by one unit, but is of practical importance in demonstrating the difficulty in using an end-point other than the 50 per cent end-point.

Materials and Methods

Virus.—The strains of Newcastle disease virus used in this series of studies are as follows: (1) Strain B isolated from a natural outbreak by Dr. F. R. Beaudette of the New Jersey Experiment Station; (2) strain W isolated by us from a natural outbreak of the disease in a flock of chickens in Bound Brook, New Jersey; (3) strain Np isolated by Dr. J. R. Beach in California; (4) Cg179, a laboratory passage virulent strain also isolated by Dr. Beach in California. This strain differs from the others in that it is able to kill 3-month-old chickens in high dilutions following intramuscular inoculation and agglutinates red blood cells poorly. Neutralization tests on embryos with strain W against classical sera furnished by Dr. Brandley demonstrated the immunological similarity. Intramuscular injection of chickens with strain B immunized chickens to Cg179, and agglutination inhibition tests with convalescent sera

from recovered birds inoculated with Cg179 showed the cross-relationship. All four strains are characterized by a filamentous shape when dried from salt solutions (1), produce a high titer of infectivity in the allantoic fluid of embryos, kill the embryo in 2 to 3 days, and often produce a characteristic hemorrhagic pattern which involves the brain and feather follicles.

Method of Titration.—Suspensions of virus were titered after preliminary low speed centrifugation at 5,000 R.P.M. for 5 minutes. Serial tenfold dilutions were made in tubes containing 4.5 cc. of cold buffered saline which had been kept at refrigerator temperature just prior to the titration. Immersion in ice water was omitted because of the stability of this virus. Ten to 12-day-old embryonated eggs opened by making a window in the side of the shell were inoculated with 1 drop of the dilute suspension. All eggs were obtained from the

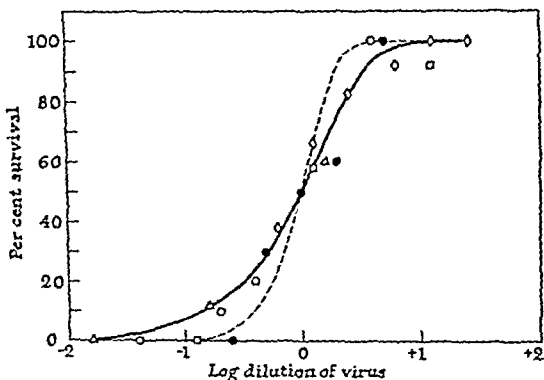


FIG. 1. End-point titration curve of Newcastle virus. Points on this curve were obtained by inoculation of ten embryos at each dilution. A 50 per cent end-point was calculated. The actual per cent of embryos surviving at a given log dilution above or below this calculated end-point is plotted against the log difference between the calculated 50 per cent end-point and the dilution inoculated. Symbols of the same kind represent one experiment. We have combined the results of five experiments to obtain the data. The solid curve is the theoretical curve for infections produced by one infectious unit. The dotted curve is for three units.

Rockefeller Institute stock which has been repeatedly found free of Newcastle disease as shown by the lack of immunity demonstrated by red cell agglutination inhibition tests and inoculation of virulent strains of the virus.

Embryos were usually incubated at 35° after inoculation (see below) and the occasional embryo dying within the first 12 to 18 hours was considered as killed by trauma and was discarded. With dilute suspensions of virus, death of the embryo begins about 2½ days after inoculation and may continue through 3 or 4 days. Incubation at higher temperatures produces more rapid multiplication of the virus and consequently a more rapid death. All surviving embryos were kept for 6 days after inoculation before discarding.

Smears were made as routine from dead embryos for microscopic examination to eliminate the occasional death occurring from accidental contamination. Previous work with gonococcus prophylaxis in embryos (13) has shown that this method picks up the great majority of such infections. However in critical experiments, blood plate cultures were also made. Since the virus of Newcastle disease agglutinates red cells, we usually checked the ability of the allantoic fluid from dead embryos used in a titration to do this. There are, however, several difficulties involved. First, concentrated suspensions of unpurified virus often show a prozone effect—possibly due to an inhibitor in the allantoic fluid. Therefore, it is necessary

to check two or three tenfold dilutions of the allantoic fluid before stating that it is negative. Secondly, different strains vary in their ability to agglutinate. Strain Cg179, our virulent laboratory strain, is a poor agglutinator producing irregular agglutination of chicken red cells only up to dilutions of 1/100 or so. Thirdly, embryos inoculated on the chorioallantoic membrane do not always develop sufficient virus in the allantoic fluid to agglutinate red cells. Thus, we cannot consider deaths which occurred at the right time and in the absence of bacteria, as not being due to Newcastle virus if they did not show red cell agglutination. However, several of the five embryos in a dilution would usually, if they died, have sufficient virus to agglutinate chicken red cells, and we could therefore conclude that the virus was present in that particular dilution.

It is well to emphasize that similar accuracy to that obtained here cannot be expected if the source of embryos is not from a clean flock of chickens, and if the above considerations are not taken into account. Particular care should be taken to demonstrate that deaths from other causes not following inoculation are not occurring in the eggs between the 10th

TABLE I
Duplicate Titrations of Various Suspensions

50 per cent end-point mortality. 3 dilutions—5 embryos each dilution

Virus	Duplicate titers		
	Titer 1	Titer 2	Difference
B—allantoic fluid.....	10 ^{-7.4}	10 ^{-7.2}	-0.2
B— " ".....	10 ^{-8.7}	10 ^{-8.6}	-0.1
Cg179—" ".....	10 ^{-8.5}	10 ^{-8.4}	-0.1
Cg179—" ".....	10 ^{-7.8}	10 ^{-8.0}	+0.2
B— " ".....	10 ^{-8.7}	10 ^{-8.5}	-0.2
B—embryo suspension.....	10 ^{-6.2}	10 ^{-5.8}	-0.4
B—purified preparation.....	10 ^{-7.6}	10 ^{-7.8}	-0.2
Same titrated in water.....		10 ^{-7.8}	

and 18th days of incubation. This may be a common source of error when using eggs bought on the open market.

RESULTS

Table I presents the results of duplicate titrations on different preparations carried out during the course of our investigation. None of these seven tests differed from the duplicate by more than 0.4 log. We therefore believe that a difference of 0.6 log is probably significant and of 1.0 log almost certainly significant. This would mean that we probably could detect a loss of 75 per cent of activity and almost certainly a loss of 90 per cent activity. A variety of other factors might be of significance in titering Newcastle virus in the embryo and we here present data bearing on some of them.

Temperature.—It has been shown for a number of viruses that the optimum temperature range is fairly narrow. For influenza it is about 35–36°C. This suggests that titrations might better be carried out at that optimum. How-

ever, the optimum temperature range for growth of Newcastle virus seems to be much greater (14). The effect of different temperatures of incubation after inoculation of the embryos with the definitive dilutions was studied by performing titration as usual, but ten instead of five embryos were inoculated at each dilution. One-half of the embryos in each dilution were incubated at one temperature and the other half at another. The 50 per cent end-points were calculated separately and are compared in Table II.

TABLE II

Effect of Temperature of Incubation on Titer Obtained from Same Preparation of Virus

Age of embryo used	Temperature of incubation	
	35°C.	39°C.
10 days.....	$10^{-6.5}$	$10^{-6.3}$
14 days.....	$10^{-6.5}$	$10^{-6.7}$

TABLE III

Effect of Route of Inoculation on Titration Results

Virus	On chorioallantoic membrane	In allantoic sac	In amniotic sac	In yolk
Cg179.....	$10^{-8.2}$	$10^{-7.8}$	$10^{-8.2}$	$10^{-9.0}$ $10^{-9.3}$ $10^{-6.8}$
Cg179 6-day-old fluid.....	$10^{-7.8}$	$10^{-8.2}$		
B strain suspension embryo.....	$10^{-6.7}$	$10^{-7.2}$		
B strain allantoic fluid.....	$10^{-9.5}$	$10^{-9.5}$		
B strain concentrated.....	$10^{-9.2}$			
B strain 46 hr. growth.....	$10^{-8.5}$			
Cg179 stock.....	$10^{-7.5}$			

Route of Inoculation.—With different strains of mumps it is necessary to introduce the inoculum by different routes to get the highest degree of takes (15). The virus of swine influenza is likely to kill the embryo if injected into the allantoic sac (16), but if inoculated by itself on the chorioallantoic membrane, relatively few of the embryos die (17); many throw off the infection and survive. It might therefore be expected that the route of inoculation would play some rôle in determining the 50 per cent end-point. A number of tests were made, again by inoculating the same dilution fluid by different routes into several series of embryos.

Table III shows the results from a series of such tests.

The inoculation on the chorioallantoic membrane was performed by first lowering the membrane through an artificial window in the side, then dropping 1 drop of the dilution tested on the membrane, and sealing with scotch tape. Allantoic inoculation was usually performed

by injection of about 0.05 cc. through a small hole in the otherwise intact egg with a short hypodermic needle. Amniotic inoculation was by direct inoculation with the visualization of the amniotic sac through an artificial window in the side. A pair of fine forceps held the edge of the amniotic membrane. Yolk sac inoculation was by a long needle ($1\frac{1}{4}$ inches) inserted through the blunt end of the egg.

Table III shows that there is no consistent difference between the methods of inoculation and that they all yield comparable results. This is in agreement with other recent reports (18). We continue however to prefer for this virus the inoculation on the chorioallantoic membrane because we believe that this method allows a more careful check of accidental deaths and gives slightly more consistent results.

Effect of Age of Embryo.—The effect of the age of the embryo used in measuring virus activity was studied by inoculating the same dilutions on two sets of

TABLE IV

Influence of Age of Embryo Used in Titration on the Calculated 50 Per Cent End-Point

Experiment No.	Age, days					
	10	11	13	14	15	16
1	$10^{-8.3}$				$10^{-8.3}$	
2	$10^{-6.5}$			$10^{-6.5}$		
3		$10^{-7.8}$		$10^{-7.5}$		
4	$10^{-9.2}$		$10^{-8.3}$			$10^{-7.8}$

embryos (chorioallantoic membrane) of different ages. The results of this are shown in Table IV. All inoculations included in this table were with strain B.

Three of the four experiments failed to show any effect of the age of the inoculated embryo. The fourth did show a probably significant effect of age. We, therefore, prefer 10- or 11-day embryos for titrations, but 12-day embryos may also be used, and when used in these experiments their use has been so specified.

Effect of Red Blood Cells.—Several of the viruses which agglutinate red blood cells are absorbed on the chick red cells in large amounts. It might, therefore, be expected that accidental contamination of the fluid with red blood cells during harvesting would considerably affect the titer obtained. However, actual tests indicate that at room temperature a minimum amount of virus is absorbed. In order to give a maximum chance for absorption the following experiments were done with the B strain which is a good agglutinator. The allantoic fluid virus was diluted to $\frac{1}{100}$ and to this a sufficient amount of red blood cells to make a 1 per cent suspension was added. Since agglutination is better at ice box temperature (19), the virus suspension with red cells was placed in the refrigerator for varying periods of time. The chicken red cells

were then spun down and the supernatant fluid titrated for virus activity by inoculation of serial tenfold dilutions. Controls were similarly treated except for the absence of red blood cells. The results are shown in Table V.

There is a consistent reduction in the embryo infectivity. This reduction is only occasionally statistically significant. These results would agree with those of Florman in indicating absorption of the virus on the red cell, but indicate clearly that a few red cells gaining entrance into the allantoic fluid when harvesting are not an important source of error.

TABLE V
*Effect of Addition of 1 Per Cent Chicken Red Blood Cell Suspension
to 1/100 Dilution of Virus*

Allantoic fluid virus	Time after red cells added, min.						Control
	Immedi- ately	7	20	30	45	60	
Diluted 1/100.....					$10^{-8.5}$		$10^{-8.7}$
" 1/100.....				$10^{-7.0}$			$10^{-7.5}$
Purified by centrifuga- tion. Dilution 1/10..	$10^{-9.0}$	$10^{-8.5}$	$10^{-8.8}$			$10^{-9.2}$	$10^{-9.2}$

SUMMARY

The application of the 50 per cent embryo mortality to a study of the virus of Newcastle is described. It has been evaluated by a series of duplicate titrations of the same sample of virus. In seven such titrations the largest difference between the two was $10^{-0.4}$. It is therefore believed that a difference of 0.6 log is probably significant and of 1.0 log almost certainly significant. This would mean that we can almost certainly detect a loss of 90 per cent of activity.

Neither temperature of incubation nor route of inoculation in the test embryos had consistent effect on the measurement of virus activity. The effect of increasing age of the incubated embryo, from 10 days up to 16 days, is slight and inconsistent. The addition of chicken red blood cells to a dilution of virus may lower the titer of the preparation, but the change is not sufficient to be of importance in the routine handling of the virus.

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STUDIES ON NEWCASTLE DISEASE VIRUS

II. BEHAVIOR OF THE VIRUS IN THE EMBRYO

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A study of the growth and behavior of an individual virus in the developing chick embryo has two important aspects. The biologist who is endeavoring to understand the comparative pathology of various experimental infections must have detailed information on this point. The chemist or commercial immunologist who uses the egg more as a test tube needs to know under what conditions he can obtain the maximum titer of virus. Although a large proportion of the same basic information is useful to both groups, it is more from the point of view of comparative pathology that these data on the virus of Newcastle disease have been collected and will be analyzed.

Methods

The general methods used are those reported in the first paper in this series. Most of the measurements have been made by titrations of embryo infectivity, since it is not certain that the activity of the virus to agglutinate red cells is inseparable from such infectivity.

Distribution of the Virus in the Embryo

Burnet (1) demonstrated in the first studies of embryo infections with Newcastle virus that the final concentration in the allantoic fluid was higher if the embryo was inoculated in the allantoic sac, and higher in the amniotic fluid if this sac was inoculated. Since then it has clearly been shown that route of inoculation plays a large rôle in the amount of virus obtained from different parts of the embryo (2). Since this is so, any comparison of the distribution of the different viruses in the embryo must take into account the route of inoculation. Our comparison presented in Table I is useful in demonstrating that, following either inoculation of the chorioallantoic membrane or into the allantoic sac, there is about 100 times as much virus in the allantoic fluid as is present in the embryo. There is however a high concentration of virus in the embryo following such inoculations. Newcastle virus then may be placed between the encephalitis groups of viruses and the influenza group. Following membrane inoculation, the viruses of Eastern and Western encephalitis (3) and of Venezuelan encephalomyelitis (4) attain the highest titer in the embryo and chorio-

allantoic membrane. West Nile virus localizes in membrane and embryo regardless of route of inoculation (5). Japanese B encephalitis also attains a higher titer in the embryo when inoculated by yolk sac (6). Newcastle virus is like these encephalitic viruses in that it kills the embryo and has a high titer in the embryo when inoculated by membrane or allantoically. However, the concentration is not as high as that of the allantoic fluid, and Newcastle virus thus corresponds to the influenza group of viruses which, following inoculation into the allantoic sac, multiply rapidly and attain their highest concentration there. To summarize, we may say that Newcastle virus resembles the encephalitis group in its ability to spread throughout the developing egg and to attain a high concentration in the embryo, but that it resembles influenza virus in its high concentration within the allantoic fluid before death.

The distribution of a virus cannot be considered separately from the growth rate. Two factors known to have an effect on the growth rate in other embryo

TABLE I
Distribution of Newcastle Virus in 11-Day-Old Developing Eggs

Route of inoculation	Allantoic fluid	Amniotic fluid	10 per cent embryo
Allantoic sac	$10^{-9.5}$		$10^{-7.2}$
“ “	$10^{-9.2}$	$10^{-8.2}$	
“ “	$10^{-9.5}$		$10^{-6.7}$
Membrane	$10^{-7.8}$	$10^{-5.5}$	$10^{-6.2}$
“	$10^{-9.5}$		$10^{-6.7}$

infections, (1) size of inoculum and (2) temperature of inoculation, have been studied in this disease.

It has been shown that inoculation of concentrated suspensions of influenza virus, of either A or B strain, will produce a lower final titer of virus in the allantoic fluid than will the inoculation of a more dilute suspension. This is apparently part of the large problem of interference by dead virus (7). Such an effect has not been found in the case of Japanese B encephalitis (6). Present studies on Newcastle virus fail to show any effect of the size of inoculum on the amount of virus finally obtained (Table II). Indeed the larger inoculum produced a larger amount of virus earlier in the course of the infection, and in consequence killed the embryo earlier. Following minimal inocula the same high titer was obtained later and death was delayed.

Effect of Temperature on Rate of Growth

The effect of temperature of incubation on the growth of a number of viruses has been recently summarized (8) and it has been pointed out that “chick embryos are more susceptible when incubated at 35 to 37°C. than at 39°C.”

This generalization does not hold for Newcastle virus as shown in Table III. Indeed such an effect is not to be expected, for this virus must multiply in a

TABLE II
Effect of Size of Inoculum on Yield of Virus Incubated at 37° C.

Experiment No.	Time of incubation <i>hrs.</i>	50 per cent end-point of allantoic fluid			
		Inoculum			
		10 ⁻³	10 ⁻²	10 ⁻¹	10 ⁻¹
1	6	10 ^{-5.4}	10 ^{-3.4}	10 ^{-2.4}	
	24	10 ^{-8.2}	10 ^{-8.0}	10 ^{-7.3}	
	43	10 ^{-9.3}	10 ^{-9.3}	10 ^{-8.0}	
2	11½			10 ^{-4.8}	10 ^{-1.8}
	22			10 ^{-8.0}	10 ^{-5.3}
	46			10 ^{-8.5}	10 ^{-9.2}
	71				10 ^{-9.5}

TABLE III

Effect of Temperature of Incubation on the Yield of Newcastle Virus from the Allantoic Fluid of 11-Day-Old Embryos Inoculated into the Allantoic Sac

Experiment No.	Dilution of virus inoculated	Time of incubation <i>hrs.</i>	35°C.		37°C.		39°C.		40°C.		41°C.	
			Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
1	10 ⁻²	48	10 ^{-8.5}	1/800	10 ^{-9.5}	1/800	10 ^{-9.7}	1/1600				
2	10 ⁻²	40	10 ^{-8.7}	1/6400			10 ^{-8.7}	1/6400			10 ⁻⁹⁺	1/6400
3	10 ⁻¹	41	10 ^{-8.5}		10 ^{-8.3}				10 ^{-8.7}			
4	10 ⁻³	48	10 ^{-8.5}								10 ^{-9.4}	
5	10 ⁻⁴	8½	10 ^{-3.4}						10 ^{-5.0}			
		18½	10 ^{-6.5}						10 ^{-8.0}			
		26	10 ^{-7.6}						10 ^{-8.6}			

host (chicken) which normally has a rectal temperature of 40 to 41°C. Furthermore, studies of the influence of temperature on the yield of virus obtained in the influenza-encephalitis group of viruses have usually not been related to time. Encephalitis virus will grow better at 37° than at 42°C., but initial growth rates seem to be about the same (3). Vesicular stomatitis grows better at 35-36°C.

than at 39–40°C., but again initial growth rates in both 7- and 10-day embryos are about the same (9). The virus of influenza B does better at 35°C. than at either 37° or 39°C., but the initial growth rates at 35° and 37°C. are very similar (10). This suggests that the relation of temperature of incubation to the growth of viruses in the embryo is not a simple problem of optimum temperature of incubation but that complicated host-parasite relations may play a large rôle (8).

Further data on the effect of temperature of incubation on the growth rate of Newcastle virus are presented in Chart 1. It may be noted that growth at

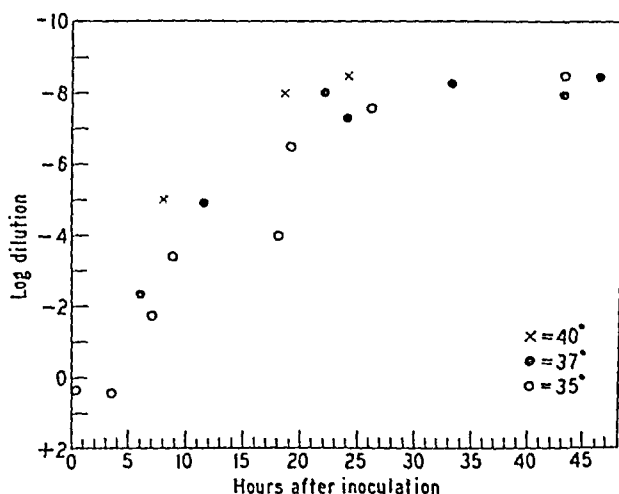


CHART 1. The relation of temperature of incubation to the growth rate of Newcastle virus in the allantoic sac of 11-day-old chicken embryos. All embryos were inoculated with a 10^{-4} dilution of freshly harvested allantoic fluid. Each point represents the titer obtained by pooling several embryos. The chart is a composite, but individual experiments (Table III, Experiment 5) demonstrate clearly the effect of higher temperatures. The titers below a log dilution 10^{-6} were obtained by inoculating 10 drops of the undiluted fluid to be tested (10^{+1} dilution).

40°C. is more rapid than at 37°C. and this in general more rapid than at 35°C., but that the final titer is about the same (Table III). As with other embryo infections (3, 4, 9–11), and some other animal virus infections initial growth through a logarithmic (12, 13) phase occurs without pathological changes, and the highest titer of virus may be obtained before pathological changes set in.

In the succeeding paper certain attempts to separate the red cell-agglutinating activity of Newcastle virus from the embryo infectivity are presented. It may be worth giving here a detailed record of the rise in red cell titer in the allantoic fluid and its relation to the infectious titer of the fluid. Henle and Henle (7) have shown in the case of influenza infections that correlations between the content of active virus in allantoic fluid and hemagglutinin titer may exist only during the stage of rapid increase of the active virus (logarithmic

phase of growth) but not after the active virus titer has reached its peak and started to decrease. Presumably this is related to loss of embryo infectivity (death) without loss of hemagglutinin activity. In comparing these results with those for the Newcastle virus we must remember that embryos infected with influenza virus frequently live for some days after inoculation and after the maximum titer of virus is obtained in the allantoic fluid. This is not equally true of Newcastle virus, for death occurs sooner after its maximum titer has been obtained. It is also more stable than influenza virus. Therefore, the relation of embryo infectivity to hemagglutinin activity after death would not be dependent upon peculiar host-parasite relations but merely would mirror the persistence of the more stable hemagglutinin characteristics as contrasted with embryo infectivity. Hence we cannot present any significant data on Newcastle virus after 48 hours (with dilute inoculum 72 hours).

Table IV presents a summary of our data on the relation of these two characteristics during the logarithmic phase of growth, following the inoculation of different concentrations of virus. When the known error in the method of determining embryo infectivity is considered (only three embryos were used in each dilution in this test) it is seen that we can conclude only that there is a concomitant increase in red cell-agglutinating activity and in embryo infectivity, and that the geometric rate of increase of hemagglutinins ceases at about the same time as does the embryo infectivity (20 to 25 hours) (Chart 1). Thus there is in our study no evidence of a separation of the two functions.

The question of a latent period before the logarithmic phase of growth begins was considered. The data in Chart 1 are inadequate to a decision. The embryos were inoculated with $\frac{1}{20}$ cc. of a 10^{-4} dilution of allantoic fluid, and this means that each embryo received about 50,000 LD₅₀ doses. This was diluted by a factor of $\frac{1}{20}$ cc. in 50 cc. (volume of egg) or $\frac{1}{1000}$, which means that a drop of test inoculum from a recently inoculated embryo would contain a maximum of 50 LD₅₀ infectious units. These calculations take no account of the probable fixation of the virus on the tissue. It follows that although in the few hours after inoculation of the virus into the allantoic sac we did not recover the "expected" amount (see Table V), we cannot conclude that there was a latent period before its proliferation. Any virus formed may have been fixed in the tissues.

Effect of Age of Embryo

In the preceding paper, it was noted that there was relatively little effect of age on the susceptibility to the virus. This, however, would not mean that the same total amount of virus was obtainable from allantoic fluid of eggs of different ages. The point was tested by the inoculation of 10-, 12-, and 14-day-old embryos into the allantoic sac (Table VI). The fluid was harvested from two to five embryos of each age, and titrations of red cell agglutination indi-

Rate of Increase of Virus in Allantoic

Time	Egg No.	Undiluted allantoic fluid									Embryo infectivity of pool	Egg No.	Red cell		
		Red cell agglutinin of harvested allantoic fluid											1/10	1/100	1/200
		1/10	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800					
hrs.															
6	1	0	0	0	0	0	0	0	0	0	10 ^{-5.3}	1	0	0	0
	2	0	0	0	0	0	0	0	0	0		2	0	0	0
	3	0	0	0	0	0	0	0	0	0		3	0	0	0
17	1	0	+	++	+++	++	+	0			10 ^{-5.3}	1	+++	0	0
	2	0	+++	+++	+	0	0	0				2	+	0	0
	3	0	+++	+++	++	0	0	0				3	+	0	0
24	1	0	0	+++	+++	+++	+	0			10 ^{-5.3}	1	0	+++	+++
	2	0	0	+++	+++	+++	++	0				2	0	++	++
	3	0	0	+++	+++	+++	++	0				3	0	++	+++
32	1	0	0	++	+++	+++	++	0	0	0	10 ^{-5.3}	1	0	+	+++
	2	0	0	+++	+++	+	0	0	0	0		2	0	0	+++
	3	0	0	+++	+++	+++	+++	0	0	0		3	0	0	+++
	4	0	+++	+++	+++	+++	++	0	0	0					
43	1	0	+	++	+++	+++	++	0	0	0	10 ^{-5.3}	1	+++	+++	+++
	2	+	+++	+++	+++	+++	+++	++	0	0		2	0	0	+++
	3	+++	+++	+++	+++	+++	+++	+	0	0		3	0	0	+++
	4	0	+++	++	0	0	0	0	0	0					
52	1	+++	+++	+++	+++	+++	+	0	0	0	10 ^{-5.3}	1	+++	h	h
	2	-	-	+++	+++	+++	+++	0	0	0		2	+++	+	+
64												3	0	0	++
												1	++	++	+++
												2	0	0	+++
												3	++	++	+++

Various Amounts of Inoculum

Inoculation					1/10,000 dilution										
Inoculated allantoic fluid					Embryo infectivity of pool	Egg No.	Red cell agglutinin of harvested allantoic fluid								Embryo infectivity of pool
1/1000	1/1600	1/3200	1/6400	1/12800			1/10	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	
0	0	0	0	0	$10^{-3.4}$	1	0	0	0	0	0	0	0	0	$10^{-3.4}$
0	0	0	0	0		2	0	0	0	0	0	0	0	0	
0	0	0	0	0		3	0	0	0	0	0	0	0	0	
0	0	0	0	0	$10^{-3.4}$	1	0	0	0	0	0	0	0	0	$10^{-3.4}$
0	0	0	0	0		2	0	0	0	0	0	0	0	0	
0	0	0	0	0		3	0	0	0	0	0	0	0	0	
+	0	0			$10^{-3.5}$	1	0	0	0	0	0	0	0	0	$10^{-3.5}$
+	0	0				2	0	0	0	0	0	0	0	0	
++	++	0				3	0	0	0	0	0	0	0	0	
++	0	0	0	0	$10^{-3.5}$	1	+	0	0	0	0	0	0	0	$10^{-3.5}$
++	+	0	0	0		2	+++	+	0	0	0	0	0	0	
+	0	0	0	0		3	0	++	+++	+++	+++	0	0	0	
++	++	0	0	0	$10^{-3.4}$	1	+++	+++	+++	+++	+++	+	+	0	$10^{-3.4}$
++	+++	++	0	0		2	+++	+++	+++	+++	++	+	0	0	
++	+++	+	0	0											
b	+++	+++	++	0	$10^{-3.4}$	1	0	0	++	+++	+++	++	0	0	$10^{-3.4}$
++	+++	+	0	0		2	0	0	+++	+++	++	0	0	0	
++	+	+	0	0											
++	+	0	0	0	$10^{-3.4}$	1	0	0	0	+++	+++	+++	+	0	$10^{-3.4}$
++	+++	+	0	0		2	0	0	0	+	+	++	+	0	
++	+++	0	0	0		3	0	0	0	+	++	+++	+	0	

TABLE V

Growth of Virus after Inoculation of a 10^{-4} Dilution of Allantoic Fluid at 35° C.

Time	Titer
hrs.	
1½	$10^{+0.3}$
3½	$10^{>0}$
7	$10^{-1.7}$
18	$10^{-4.0}$

TABLE VI

Effect of Age of Embryo on Titer of Virus Obtained from Allantoic Fluid

10 days		12 days		14 days	
Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
$10^{-9.0}$	1/4000	$10^{-9.0}$	1/2000	$10^{-9.0}$	1/1000

cated that there was a slight decrease in the amount of virus obtainable. This was not great enough to be picked up on infectivity measurements.

SUMMARY

The virus of Newcastle disease of chickens resembles those of the encephalitis group in its ability to spread throughout the developing egg and embryo, but it is similar to influenza virus in the high concentration of it found in the allantoic fluid before death. No effect of the size of the inoculum on the final titer of virus in the allantoic fluid was detected. Good growth occurred at temperatures from 35° to 41°C., apparently more rapid at 40°C. than at 35°C. No appreciable development of virus capable of agglutinating red cells but of low embryo infectivity was found. Although virus multiplication was not immediately perceptible after inoculation, this cannot on present evidence be attributed to a real lag phase.

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STUDIES ON NEWCASTLE DISEASE VIRUS

III. CHARACTERS OF THE VIRUS ITSELF WITH PARTICULAR REFERENCE TO ELECTRON MICROSCOPY

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PLATES 8 AND 9

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Electron micrographs of Newcastle virus suspended in allantoic fluid or water show a number of irregular globular forms (1). Filamentous or tailed forms have not been found in these fluids. However, when the virus is transferred to saline and a mount is made directly of this material, tailed or sperm-like forms are found to predominate (2-4).

The identification of certain particles seen in the electron microscope as virus particles is not easy. Even some 10 years after the original description of the tobacco mosaic virus as a long thin rod (5) there is some discussion as to whether these particles represent individual units (6) or aggregates of virus (7). It is not legitimate to accept any spherical particle obtained from a mixture of materials as virus just because it has a size which agrees with previous calculations and because it is frequently found in these partially purified preparations. All available methods for associating or dissociating the two must be applied.

We first would like to outline certain evidence which we believe associates the filamentous or sperm-like bodies seen in saline preparations with the individual virus particles. We will then take up the evidence that the tailed forms are derived from the globular forms in the allantoic fluid.

The Evidence for an Association of Virus Activity with the Filamentous Units

The evidence indicative of an association of filamentous units will be considered under several heads:—

1. *Lack of Similar Forms in Other Infections of the Embryo.*—Several other virus infections have been studied under the electron microscope. The most fully reported is the influenza group (8, 9), in which certain filamentous forms have been described (10). However, these forms are in the minority in the preparations that have been published and have been pictured as long solid threads, never as sperm-like forms of variable thickness. They have not definitely been identified as virus particles. The virus of cat pneumonia has recently been studied in preparations from the allantoic fluid of chick embryos and no filamentous forms are described (11). Eastern and Western encephalomyelitis viruses are purified in higher centrifugal fields but infected chick embryos are used as starting material. No filamentous forms have been de-

scribed (12). We have studied partially purified preparations of swine influenza, mumps, and Eastern equine encephalomyelitis without seeing any forms which resemble the usual picture of Newcastle virus when resuspended in saline. This negative evidence makes it unlikely that the peculiarities observed are ordinary artifacts.

2. *Period at Which Filamentous Forms Are First Obtained.*—Filamentous forms have been obtained from allantoic fluid as early as 24 hours after inoculation. This corresponds to the time of first increase in virus titer of the allantoic fluid (13). The forms are obtainable from that time on until death of the embryo some 24 to 48 hours later, during which period the titer is maintained. They have been found in preparations incubated at 35°, 37°, and 41°C. They are thus obtainable at all times when the virus titer is high. Their presence early in the infection indicates that they are not a degenerate form.

3. *The Forms Are Obtained from Four Different Strains.*—The four strains used in the studies here reported have all shown predominantly a tailed or filamentous form when suspended in saline and dried (Figs. 2, 4, 6). None of them has shown this form in allantoic fluid (Figs. 1, 3, 5). The presence of the tailed form in saline preparations has been reported in another strain from the United States (3) and in the classical Doyle strain (4).

4. *The Forms Are Agglutinated by Specific Antisera.*—The infection of a vertebrate host with a pathogenic organism usually results in antibodies against that organism. On previous evidence we can expect that an organism which has been identified as the causative agent of a disease may be agglutinated by convalescent sera from that disease. Failure for this to happen militates against the identification of the material under test as the infectious agent. Agglutination by convalescent sera is however not conclusive evidence for identification of agent, and disease, as witness the rise of the proteus OX titers in various rickettsial fevers, and the increase of anti-beef heart particle titer in syphilis. An increase in the agglutinating titer against vaccinia particles in rabbits infected with vaccinia (14) and of fowl pox bodies of chickens infected with fowl pox (15) has been demonstrated. Such tests have not hitherto been applied to electron microscope studies.

In the course of the present work, agglutination of Newcastle virus particles was first demonstrated in a $1/6$ dilution of antiserum against the classical strain of the disease.¹ This was controlled by tests with serum from a rooster hyperimmunized with chick embryo preparations of equine encephalomyelitis.

A drop of fluid from each mixture was mounted on an electron microscope screen 15 minutes, 2 hours, and 5 hours after the test was started. Slight beginning clumping at 15 minutes, which became definite in 2 hours, was noted with the microscope in the Newcastle immune sera. The control sera failed to agglutinate even in 5 hours. That the agglutination was not due to a reaction with some normal tissue component of the virus is made likely by the fact that the

¹ I am indebted to Dr. C. A. Brandley for furnishing these sera.

anti-Newcastle sera in the same test failed to agglutinate partially purified influenza virus from chick embryos.

The agglutination test was repeated in another way.

Of four chickens which had been used in the terminal dilution of a titration, two had developed good red cell agglutination inhibition titers (presence of infection) and two failed to develop antibodies (no infection). To $\frac{1}{2}$ cc. of serial two fold dilutions of serum, 0.1 of a cc. of virus concentrate was added. The concentrate had been prepared by centrifuging the virus from the allantoic fluid and resuspending in saline equal to 0.1 the original volume of allantoic fluid. The combination was put in the refrigerator overnight.

As shown in Table I the sera from the infected chickens agglutinated macroscopically the virus suspension in dilutions of $\frac{1}{16}$ and $\frac{1}{32}$. The control sera failed to agglutinate. Electron microscope pictures prepared 1 hour after the

TABLE I
Macroscopic Agglutination of Newcastle Virus Suspension

Sera and virus concentrate in saline were put in refrigerator overnight.

Chicken sera used	No.	Dilution of sera					
		1/4	1/8	1/16	1/32	1/64	1/128
Known positive sera	1	+++	+++	+++	++	0	0
	2	+++	+++	++	0	0	0
Known negative sera	1	0	0	0	0	0	0
	2	0	0	0	0	0	0

addition of the sera showed, in the $\frac{1}{4}$ and $\frac{1}{8}$ dilutions, masses of clumped virus with no discrete particles left. The $\frac{1}{16}$ dilution of both positive sera showed some free particles. No microscopic agglutination (electron microscope) was seen in any dilution of the controls.

5. *Association of Infectivity and Red Cell-Agglutinating Activity with Particles of about 100 μ .*—It was early demonstrated by filtration experiments that Newcastle virus has a diameter between 80 and 120 μ (16). It was to be expected then that centrifugation at a force sufficient to concentrate influenza virus would throw down Newcastle virus. This was found to be so (2). Indeed it has been demonstrated (3) that a force of 15,000 to 20,000 g for 15 to 20 minutes is sufficient to sediment the virus particles (as determined by red cell agglutination). This accords with the size of the particles seen by electron microscopy (17).

For the sake of completeness, we present data on three other strains of the virus, demonstrating the ease of sedimentation and concentration. These tests were carried out in a higher gravitational field than necessary and therefore do not indicate as clearly as does the work of Cunha *et al.* (3) that the size of the infectious particle is about 100 μ (Table II).

Although the red cell-agglutinating activity of the influenza-mumps-Newcastle group of viruses is much more stable than the infectivity of the virus for the embryo, it has not been possible as yet to separate the red cell agglutinating factor from the 100 m μ particle. In an attempt to do this we studied the effect of freezing and thawing on the sedimentability of the virus (measuring both red cell and embryo infectivity). It was consistently found that freezing and thawing of a preparation of infected allantoic fluid caused a much larger amount of virus to remain suspended in the supernatant fluid after centrifuging at 24,000 R.P.M. for 40 minutes (30,000 g minimal) (Table III). Both the infectivity and red cell-agglutinating titers of the top layer of supernatant were about ten times greater when the material had been frozen and thawed beforehand. There are several possible explanations of this phenomenon. (It is, of course, understood

TABLE II

*Sedimentation of Several Strains of Newcastle Virus*Centrifugation at 24,000 R.P.M. (30,000 g⁺) for 30 minutes.

Strain	Original fluid		First supernatant		Pellet resuspended after second centrifugation	
	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
Cg179		1/40		0	10 ^{-8.8}	1/80
	10 ^{-7.8}		10 ^{<3.5}		10 ^{-8.1}	
	10 ^{-8.4}	1/10	10 ^{-5.7}	0	10 ^{-8.2}	1/80
Np	10 ^{-8.8}	1/1600		0	10 ^{-8.9}	1/800
W		1/400		1/10	10 ^{-8.0}	1/400

that the flocculi and clumps of virus and protein due to freezing and thawing have in each instance been broken up by repeated pipetting).

It is conceivable that the inherent viscosity of the suspending medium has been increased by freezing and thawing. This might change the character of the convection currents in the tube and might also directly affect the sedimentation rate of the virus. Such a state of affairs is unlikely because after a second centrifugation at 24,000 R.P.M. in which the virus was in saline the supernatant from the preparation—which had originally been frozen and thawed,—still contained a higher hemagglutinating titer than the control supernatant. Another explanation is that the virus has in some way been altered so that it does not sediment as well. This could be consequent either on the formation of smaller particles (a breaking up of perhaps 10 per cent of the virus particles) or on the change from the compact spherical to the larger filamentous shape, which seems to occur when the virus is transferred from allantoic fluid to saline solution (1).

Such a change might also occur in unfrozen preparations after standing. However, an apparent change in size after freezing and thawing may not indicate the breaking up of a proportion of the individual particles, but instead the separation of agglomerate masses. This would explain the frequent increase in total titer of virus obtained when purifying this virus (2) and that of influenza (18).

6. *Infection Is Produced by Very Few Particles.*—If infection of the host is produced by an inoculum containing a very few particles, perhaps only one, it is likely that a single particle is the infecting agent (19). If, however, the mini-

TABLE III
Effect of Freezing and Thawing on Sedimentability of Newcastle Virus

Experiment	Original		Supernatant		Pellet resuspended in same volume saline after 1 centrifugation 40 min.	
	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer	Embryo infectivity	Red cell titer
1 Unfrozen	$10^{-8.6*}$	1/3200	$10^{-6.5}$	1/24	$10^{-8.5}$	1/3200
Frozen			$10^{-6.9}$	1/384	$10^{-8.2}$	1/3200
2 Unfrozen				1/24	$10^{-8.3}$	1/3200
Frozen				1/384	$10^{-8.2}$	1/3200
3 Unfrozen			$10^{-6.2}$	1/32	$10^{-8.2}$	1/800
Frozen			$10^{-7.2}$	1/512	$10^{-8.2}$	1/800
4 Unfrozen	$10^{-9.0}$	1/3200	$10^{-4.5}$	1/16		1/3200
Frozen slowly	$10^{-8.8}$	1/3200	$10^{-6.8}$	1/128		1/3200
Frozen quickly	$10^{-8.2}$	1/3200	$10^{-7.5+}$	1/1024		1/1600

* This material titered to $10^{-8.6}$ after three freezing and thawing treatments.

imum infecting dose contains thousands or millions of particles of the calculated size, there is more room for error in identifying the particle as the infecting agent.

The evidence that a very few particles of Newcastle virus produce infection and death of the embryo is threefold. (a) Calculation of the number of particles in a suspension, based on the size of the particle in the electron microscope, the light scattering, and the nitrogen content of the suspension, shows that there are only a few more particles than there are infectious units. The difference between the two measurements is well within the limits of experimental error. (b) Equally high titers may be obtained in two different hosts, and (c) end-point titration curves agree with the single particle infection theory. None of these lines of evidence is in itself conclusive but taken together they make it

likely that the particles seen under the electron microscope are the virus. To deal with these points individually:

(a) Electron microscope measurements of the size of head of the large fairly uniform particles in the purified preparation average $83 \text{ m}\mu \times 146 \text{ m}\mu$. Light-scattering measurements (20) combined with nitrogen determination on washed virus lead to an estimate of 115 to 120 $\text{m}\mu$ in size,² and a molecular weight of about 500 million. In a suspension containing 1 gm. of protein, there would then be roughly 1.2×10^{-15} particles. It has, however, been shown that 1 gm. of virus (assuming $N = 1/10$ of total mass) will titer to $10^{-14.6}$ (50 per cent end-point). There must then be about five particles present at the 50 per cent end-point.

(b) Parker (19) has pointed out that when a given virus suspension attains the same high titer and reaches the same end-point in three different hosts (vaccinia in rabbit, mouse, and chick embryo), it is probable that infection is produced by one particle. The strain Cg179 which is highly virulent for chickens was therefore simultaneously titered in 10-day chick embryos and young chickens (Table IV). There

TABLE IV
Titer of Cg179 on Chick Embryos and in Chickens

Preparation No.	10 days embryos	2 wks. chicks	2 mos. chickens
1	$10^{-8.8}$	$10^{-8.6}$	
2	$10^{-8.1}$	$10^{-8.2}$	
3	$10^{-7.2}$		$10^{-6.8}$
4	$10^{-7.5}$		$10^{-6.7}$

was no detectable difference in result on the 10-day chorioallantoic membrane and on the 2 weeks-old-chicken. There was a slight decrease (of questionable statistical significance) when 2 months-old-chickens were inoculated. We have then two hosts of roughly equal susceptibility to high dilutions of virus.

(c) The theory that infection is produced by one infectious unit has been extensively discussed (21) and the expected curve for such a case outlined. Our results (22, Chart 1) agree with this predicted curve. This type of curve at best merely indicates that when infection has been produced in the embryo it may have been initiated by one particle. It does not take into account the virus particles which have been lost. These amount to thousands in the case of tobacco mosaic virus.

Points (b) and (c) are brought into the discussion not because of any belief that they prove the identity of the demonstrated particle and infectious unit, but because they agree with the evidence in (a) that infection is produced by a very few particles.

Apparent Change in Shape of Infectious Particle

If we accept the sperm-like or filamentous forms of Newcastle virus, when prepared from saline suspensions, as representing actual virus particles, how

² Actual measurements and calculations of size were carried out by Dr. Roger Herriott.

may we then explain their absence in the original allantoic fluid? Saline suspensions in which the virus is not concentrated show many typical tailed forms, whereas none are seen in allantoic fluid having this same titer of virus. We believe that the virus particle has another form in the allantoic fluid and that under the influence of saline it is changed into the forms just mentioned. The evidence in favor of this is as follows:—

1. Filamentous forms are absent from the allantoic fluid.
2. Filamentous forms can be produced by simple dialysis against saline. This is accomplished without loss of activity.
3. Suspensions maintained in different pH solutions give different forms.
4. The form of virus suspended in a phosphate buffer at pH 8 differs from that suspended in a borate buffer at the same pH.
5. Spherical forms can be obtained from allantoic fluid by resuspending in water. These will show filamentous types under the electron microscope if increasing concentrations of saline are added.
6. Partial inactivation of spherical forms in water by heat, formaldehyde, or mustard gas prevents the development of the filamentous forms.

It may be worth while to detail the experimental evidence on each of these points.

1. Absence of Filamentous or Tailed Forms from Allantoic Fluid.—Numerous unsuccessful attempts were made to see these forms in the unconcentrated allantoic fluid.

To preclude the possibility that the mechanical treatment of centrifugation and resuspension might cause the change in shape, normal allantoic fluid was substituted for saline during both cycles of centrifugation of one strain. No tailed forms were found after a careful search of many fields. The same concentration of virus in saline had produced great numbers of tailed forms in each field. This contrast between allantoic-fluid virus and saline-washed virus held true of all four strains.

2. Production of Filamentous Forms by Dialysis against Saline.—Filamentous forms may be observed to develop within the original allantoic fluid when it is dialyzed against saline solutions. These forms do not have the clear cut morphology of the typical tailed virus which has been centrifuged and transferred to saline, but do show a definite change in morphology. In the first experiment, infected allantoic fluid was put in a cellophane sac under slight pressure and fluid withdrawn at intervals of several hours. Filamentous forms were found during the first 12 hours. In the two subsequent experiments the virus was dialyzed against saline and water. The infectiousness of the preparations was determined and electron microscope screens prepared at intervals. The data are summarized in Tables V and VI.

We have concluded that a definite change in morphology may be brought out by dialysis against saline with little loss of infectiousness, but since other methods of investigating this morphological variation proved capable of bringing out sharper differences, we did not pursue this aspect further.

3. Effect of pH.—When virus is transferred from allantoic fluid to a saline solution by centrifugation and resuspension, it is transferred from a medium of roughly pH 8 to one of roughly pH 7—especially if the saline is buffered by phosphate. It was im-

portant then to determine the effect of various hydrogen-ion concentrations on the morphology and activity of the virus.

Infected allantoic fluid was centrifuged in the usual manner and the virus resuspended in one-tenth of the original volume of normal saline. This then was brought up to the original volume of normal saline. This then was brought up to the original volume in the particular buffered solution desired by a one to ten dilution. pH recordings are actual determinations with Coleman pH electrodes dipped in the virus solution.

TABLE V
Dialysis against 0.15 M Saline
No pressure

Effect on	Original	Time, hrs.					
		2	10	50	70	94	216
Morphology.....	Spherical $10^{-8.5}$	Spherical	Fuzzy		Stringy		
Titer.....			$10^{-8.8}$	$10^{-8.5}$	$10^{-8.4}$	$10^{-8.5}$	$10^{-8.4}$

TABLE VI
Dialysis through Cellophane Sac
Original titer $10^{-9.5}$. No pressure

Dialyzed against	Effect on	Time, hrs.			
		6	24	72	216
Normal saline	Morphology	Fuzzy and stringy	Indistinct tailed forms		Filamentous
	Titer			$10^{-8.5}$	$10^{-8.2}$
Buffered saline	Morphology	Stringy			
	Titer		$10^{-8.8}$		
Water	Morphology	Large irregular dots		Large irregular dots	
	Titer			$10^{-8.4}$	

Since the changes were greater on the alkaline side of pH 7 (Table VII), the second experiment was limited to this. The data obtained are presented in Table VIII.

Several things seemed clear from these experiments on the effect of pH. First, filamentous forms were not seen in moderately alkaline solutions even though activity was well preserved. Secondly, the tailed and filamentous forms were not preserved indefinitely at any of the pH concentrations tested. Finally it also seemed clear that it was not the change from pH 8 to pH 7 in itself which caused the appearance of the filamentous forms but rather some change in salt concentration.

4. *The Effect of Phosphate Buffer.*—Further evidence of the secondary importance of hydrogen ion concentration *per se* was obtained by studying morphology and activity at pH 7.0, 7.5, and 8.0 all in phosphate buffers (previous studies on the effect of pH had used a borate buffer for pH 8). Filamentous and tailed forms predominated at all three pH's and in two different strains of virus (B and Cg179).

TABLE VII
Effect of Different Hydrogen Ion Concentrations on the Morphology and Activity of Washed Virus

pH	Effect on		Time			
			2 hrs.	1 day	12 days	19 days
3	Activity	Embryo titer Red cell titer	$10^{-4.0}$	$10^{-4.5+}$	$10^{-4.2}$ 1/100	
	Morphology			No filamentous forms seen		
5	Activity	Embryo titer Red cell titer		$10^{-7.5}$	$10^{-5.5}$ 1/800	$10^{-5.2}$
	Morphology				Few filamentous	Spherical, many with tails
7	Activity	Embryo titer Red cell titer	$10^{-4.4}$		$10^{-7.6}$ 1/800	
	Morphology		Typical filamentous	Tailed and filamentous		Spherical
8.0	Activity	Embryo titer Red cell titer		$10^{-8.8}$	$10^{-8.5}$ 1/400	
	Morphology				Spherical	No filamentous
8.8	Activity	Embryo titer Red cell titer		$10^{-4.5+}$	$10^{-5.5+}$ 1/800	
	Morphology			Filamentous		No filamentous. Outlines vague
9.2	Activity	Embryo titer Red cell titer	$10^{-6.2}$	$10^{-7.5+}$	$10^{-7.5+}$ 1/1600	$10^{-6.4}$
	Morphology		Hazy, and crystals	No filamentous	Sharply defined hard dots	Sharply defined hard dots

5. *Effect of Molarity of Salt Solution.*—Infected allantoic fluid contains a great deal of extraneous material besides the virus particles. This makes it difficult to differentiate individual particles and to identify each virus particle. For this reason we felt that the evidence for a change in form would remain obscured until it was possible to obtain spherical particles free from the original allantoic fluid. To this end we undertook to study the morphology and infectivity of Newcastle virus in distilled water. Surprisingly the virus can be centrifuged and resuspended in water without loss of

infectivity. It has a spherical shape which is readily converted into a tailed or filamentous form by the addition of 0.07 to 0.15 molar sodium chloride.

The experiments summarized in Table IX were performed by first resuspending the pellet obtained from ultracentrifugation in water. This washing in water was repeated, and the second centrifugate was resuspended in one-half to one-sixth of the original fluid volume. This water suspension was then brought back to approximately the original volume in the particular strength of saline desired. Suspensions were stored at 4°C.

TABLE VIII
Effect of pH on the Morphology and Activity of Newcastle Virus

pH	Effect on	2 hrs.	2 days	4 days	12 days	16 days
6.8	Activity Morphology	Filamentous	$10^{-8.2}$ Filamentous	Filamentous	$10^{-7.6}$ Few filamentous	Rare filamentous
8.0	Activity Morphology		Rings	Small hard dots	$10^{-7.6}$	Irregular mixture
8.8	Activity Morphology	Filamentous and tailed	$10^{-8.3}$ Spherical	Large with vague outline	$10^{-8.0}$	Large round spheres
9.2	Activity Morphology		$10^{-8.2}$ Small dots	Irregular in outline	$10^{-7.8}$	
9.3	Activity Morphology		$10^{-8.4}$ Small dots			$10^{-7.6}$ Dense round small spheres

Figs. 7 to 12 show the gradual transition from the spherical to the filamentous, with no measurable change in infectiousness, as shown in Table IX.

The ability of saline to bring about these changes in form as seen on electron microscopy immediately raises the question of effect through various osmotic pressures. It must be remembered, however, that as the drop of saline containing the virus particles dried at room temperature on the collodion film of the electron microscope screen there was an increase in salt concentration which makes it impossible to state exactly what the osmotic pressure was during the drying. However, it has been found that virus suspended in 0.15 M sucrose is just as active as the original allantoic fluid and has a spherical form on electron microscopy. It may be worth pointing out that the effect of the salt solution seems to be quick for the filamentous forms were present within 10 minutes of adding the saline plus the time taken in drying (5 to 10 minutes).

TABLE IX
Effect of Molarity of Saline on Morphology and Activity of Virus

Experi- ment No.	Time	Effect on	H ₂ O	Molarity of saline					
				0.05	0.1	0.15	0.2	0.25	0.3
1	1 day	Embryo infec- tivity Red cell agglu- tination Morphology	10 ^{-8.2} 12,800 Spherical	10 ^{-8.9} 12,800 Spherical	6,400 Spherical	10 ^{-9.0} 6,400 Tailed	6,400 Heavy fila- ments	1,600 Heavy fila- ments	10 ^{-7.2} 1,690 Clumps*
	4 days	Embryo infec- tivity Morphology	10 ^{-8.7†} Few asymmet- rical. None filamentous	10 ^{-8.7}	Mostly spherical	10 ^{-8.3} Thick fila- ments	Heavy fila- ments	Large vague forms, few tails	10 ^{-8.0} Clumps*
2	½ hr.	Embryo infec- tivity Morphology	10 ^{-8.8} Spherical	(0.07 M) Tailed		Filamentous and tailed	Filamentous and tailed	Filamentous and tailed	
	5 days	Embryo infec- tivity Morphology	10 ^{-8.3} Spherical						
3	10 min.	Morphology	Spherical§	(0.07 M) Tailed forms beginning		Filamentous forms thick	Filamentous	Filamentous and tailed	

* Clumps visible grossly.

† Determined after 8 days in water.

§ Gold-shadowing revealed a few short thick tails projecting from spheres.

This agrees with the immediate appearance of filamentous forms on transfer into saline during the original centrifuge studies described in the first part of this paper.

6. *Effect of Partial Inactivation of Spherical Forms on the Ability of the Virus to Change Shape.*—The question which was constantly in the background was, —Is this change a real one, or is it due to some peculiar distortion of the virus on drying? It was thought that if it were possible to prevent the change in shape brought about by saline by some previous treatment of the virus in the water suspension, this would make it less likely that we were dealing with an artifact. To this end, 0.02 per cent formaldehyde was added to a water suspension of virus and this suspension kept at 4°C. for 4 days. Sufficient NaCl solution was then added to produce a 0.15 M solution and immediately thereafter and 5 minutes afterwards screens were prepared for electron microscopy.

TABLE X
Effect of 0.02 Per Cent Formaldehyde on the Change of Shape Brought about by Saline

Time <i>hrs.</i>	Water suspension		0.02 per cent formaldehyde in water	
	Activity	Morphology when placed in 0.15 M saline	Activity	Morphology when placed in 0.15 M saline
0	10 ^{-8.7}	Filamentous		
1			10 ^{-8.3}	Filamentous and tailed
4			10 ^{-7.5+}	Filamentous and tailed
27			10 ^{-7.5+}	Spherical and filamentous
72			10 ^{-8.5+}	Most spherical
120		Filamentous and tailed	10 ^{-2.5+}	Most spherical; few filamentous
288	10 ^{-6.7}	Few filamentous	10 ^{-2.5}	

The typical spherical forms of the water suspension were preserved by the previous treatment with formaldehyde despite the presence of the sodium chloride. Only a rare tailed virus particle was seen on thorough search of the screen. The failure to find forms other than spherical in a preparation of virus which had been treated with formaldehyde before centrifugation was reported by Taylor (23) at the Electron Microscope Society Meeting in 1946. On the other hand, a control suspension of virus to which saline was added before placing in the refrigerator, and to which 0.02 per cent formaldehyde was added after 4 days at 4°C., showed a profusion of tailed forms. This would indicate that it is not the mere presence of formaldehyde which prevents visualization of tailed forms, but that it is necessary for the formaldehyde to act over a period of time.

This idea was tested by comparing the ability of the spherical forms to change into tailed and filamentous forms with and without 0.02 per cent formaldehyde over a period of time. The results are presented in Table X. The electron microscope screens were prepared within a few minutes after adding the saline.

It is apparent that roughly the same results were obtained. 0.02 per cent

formaldehyde did not inhibit the change in shape until after some days during which time it had a chance to begin to inactivate the virus. However, conversion seemed to be inhibited long before inactivation had proceeded very far. It is not surprising then that the control tube in water alone produced but few filamentous forms after 12 days in the refrigerator, at which time roughly 99 per cent of the virus was apparently inactivated.

One other method of inactivation was studied fairly carefully for its ability to prevent the conversion into filamentous forms. Water suspensions were heated in a water bath at 50°C. for various periods of time. Portions were then brought to 0.15 M NaCl concentration and electron microscope screens were prepared. As can be seen in Table XI, long before inactivation was complete

TABLE XI

Effect of Heating (50°C.) on Change to Filamentous and on Infectivity of Virus

Experiment No.	Time	Morphology when transferred to saline	Titre
	<i>min.</i>		
1	0	Filamentous	$10^{-8.7}$
	10	No tailed forms seen; few asymmetrical	$10^{-6.0}$
	20	None filamentous or tailed	
	30	Beginning aggregation; outlines indistinct	
	60		$10^{-1.0+}$
2	0	Excellent filamentous forms	
	1	Most forms tailed or filamentous	
	5	Few scattered filamentous forms, some with long thin tails	
	10	Hard dense forms. Outlines irregular but essentially spherical.	
	15	Few clumps Dense hard forms, most spherical. No filamentous forms	

as far as infectivity was concerned, conversion was prevented. It is well to remember here, as in the experiments on the effect of formaldehyde, that the loss of only 3 logs in a titration means a 99.9 per cent inactivation. This amount of inhibition of conversion as seen in the electron microscope would be called complete. In a second experiment the amount of heating necessary for this inhibition was more accurately determined, but no activity determinations were made. Again it was found that 10 minutes at 50°C. prevented the conversion.

Two other methods of inactivation were studied in passing. Irradiation of water suspensions with ultraviolet light for 20 minutes in one experiment and for 60 minutes in another prevented conversion. Five to 10 minutes of irradiation under the same conditions had been found to render the virus non-infectious for the embryo. Treatment with mustard gas at a concentration of 2×10^{-4} M (24) also made the virus incapable of producing the filamentous form. In general reactions which inactivated the virus were capable of preventing the conversion, perhaps to the same degree.

DISCUSSION

Two separate ideas have been presented in this paper. The evidence for the first, that certain filamentous and tailed forms represent virus particles, is fairly good. It is based on their characteristic shape, on their size and number, and on immunological evidence.

The second idea, that there is a genuine change in shape from a spherical to a filamentous one, is a more difficult one. While repetition readily establishes the reproducibility of the phenomenon, it does not discourage the idea of a reproducible artifact. This problem may be debated at length and involves the basic problem of the applicability of electron microscope findings in the biological field. Does the form or shape of the virus of Newcastle disease *in solution* depend upon the presence or absence of a certain concentration of salt? It might be thought that the best way to answer this would be by applying certain known physical methods to a study of the virus suspensions. Light-scattering measurements were made by Dr. R. M. Herriott on the virus in allantoic fluid and in saline. No change was found. Then the scattering of light was determined in purified suspensions before and after adding salt. No change was found. But we were unable to get any information which would indicate how much change in shape without change in size might occur without a change in the light scattering.

The asymmetry of particles such as the tobacco mosaic virus is easily established as occurring in solution by birefringence on streaming. In a capillary tube supported between two crossed prisms it was easy to demonstrate the marked streaming birefringence of a 1 per cent suspension of tobacco mosaic virus. This same set up failed to elicit birefringence on streaming in a Newcastle virus suspension in saline which had fifteen times the original concentration in the allantoic fluid and which was a milky white in color. But we cannot conclude from this that the filamentous forms do not exist in suspension. The electron microscope does not indicate that they in any way approach the sharp rigid rod-like shape of tobacco mosaic.

A third method of studying the shape of macromolecules in solution is to study their viscosity. According to Einstein's formula a change in shape should be accompanied by a definite change in viscosity. But in order to get determinable differences of viscosity between the solvent and the solvent plus virus, Dr. Herriott found it necessary to employ concentrations of virus ten times as great as those used in preparing the electron microscope screens. This increased concentration seems to be accompanied by such agglomeration of the individual particles that again the failure to determine a difference between the viscosity of the virus in water and in saline is not significant.

Other physical methods will have to be employed before we can make a final decision. In the meantime the reproducibility and the marked extent of the change, plus the effect of partial inactivation of the virus favor the reality of the phenomenon.

If the change in shape is found to occur in solution it may be related to an enzyme like the ribonuclease which was found to be active in saline and inactive in water solutions.

In passing it is well to emphasize that we have studied and discussed a relatively simple question. Does the virus change from a roughly spherical particle to a roughly filamentous particle under the influence of saline? This excludes the problem of pleomorphism. Our observations would rather indicate that the Newcastle virus is pleomorphic much like the pleuropneumonia bodies. The range of such variation in shape has not been studied.

SUMMARY

1. It is likely that certain tailed and filamentous particles seen on electron microscope examination of partially purified saline suspensions of Newcastle virus are the individual virus particles because:

(a) They have a highly characteristic shape not seen in other virus preparations.

(b) They are present whenever the virus is present in high concentration.

(c) Their size agrees with the size of the virus as calculated from light scattering and centrifuge data.

(d) They are agglutinated by specific antisera.

(e) Infection may be produced in the embryo by relatively few of these particles.

2. It is possible that these filamentous forms have been derived from spherical forms without loss of activity because:

(a) Such filamentous forms are not found in the original allantoic fluid when this contains a comparable amount of virus.

(b) Filamentous forms appeared in the original allantoic fluid when it was dialyzed against saline solution.

(c) Filamentous forms were produced at certain hydrogen ion concentrations but not at others, in solutions maintaining the same infectivity for the embryo.

(d) Spherical forms were obtained by suspending the partially purified virus in water instead of saline. In this the virus remained moderately stable.

(e) These round forms could apparently be converted into tailed and filamentous forms by the addition of saline, again without loss of activity.

(f) This "conversion" could be inhibited by partial inactivation of the water suspension of virus.

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Newcastle virus (strain Np) in crude allantoic fluid. $\times 18,000$.

FIG. 2. Same strain in same concentration but partially purified by centrifugation and resuspension in 0.15 M saline. $\times 18,000$.

FIG. 3. Newcastle virus (strain W) in crude allantoic fluid. $\times 18,000$.

FIG. 4. Strain W prepared from 0.15 M saline. Same concentration as Fig. 3. $\times 18,000$.

FIG. 5. Strain Cg179. After centrifugation twice and resuspension in water. $\times 18,000$.

FIG. 6. Same preparation but resuspended in 0.15 M saline. $\times 18,000$.

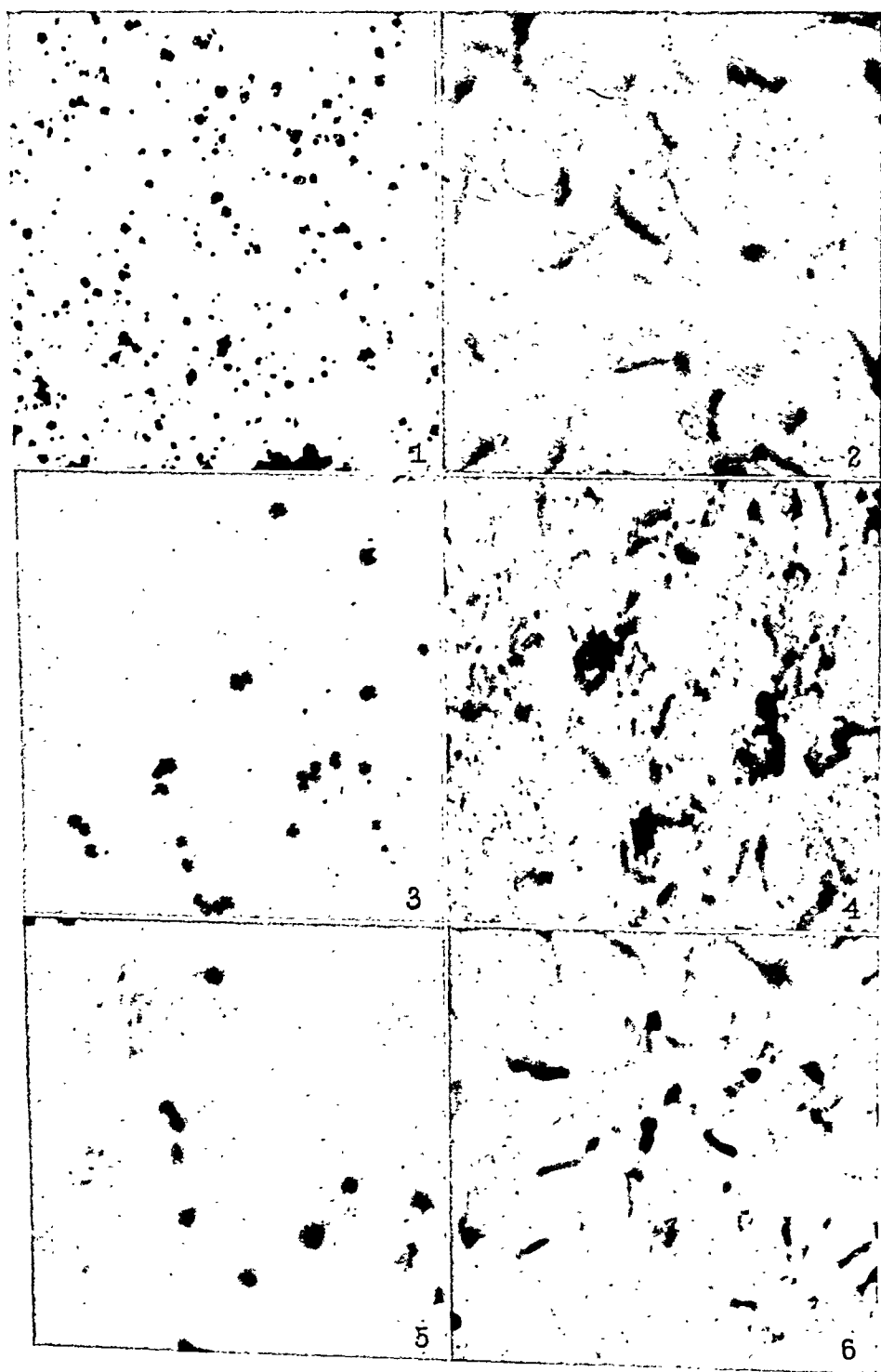


PLATE 9

FIG. 7. Newcastle disease virus (strain B) washed and resuspended in water.
× 18,000.

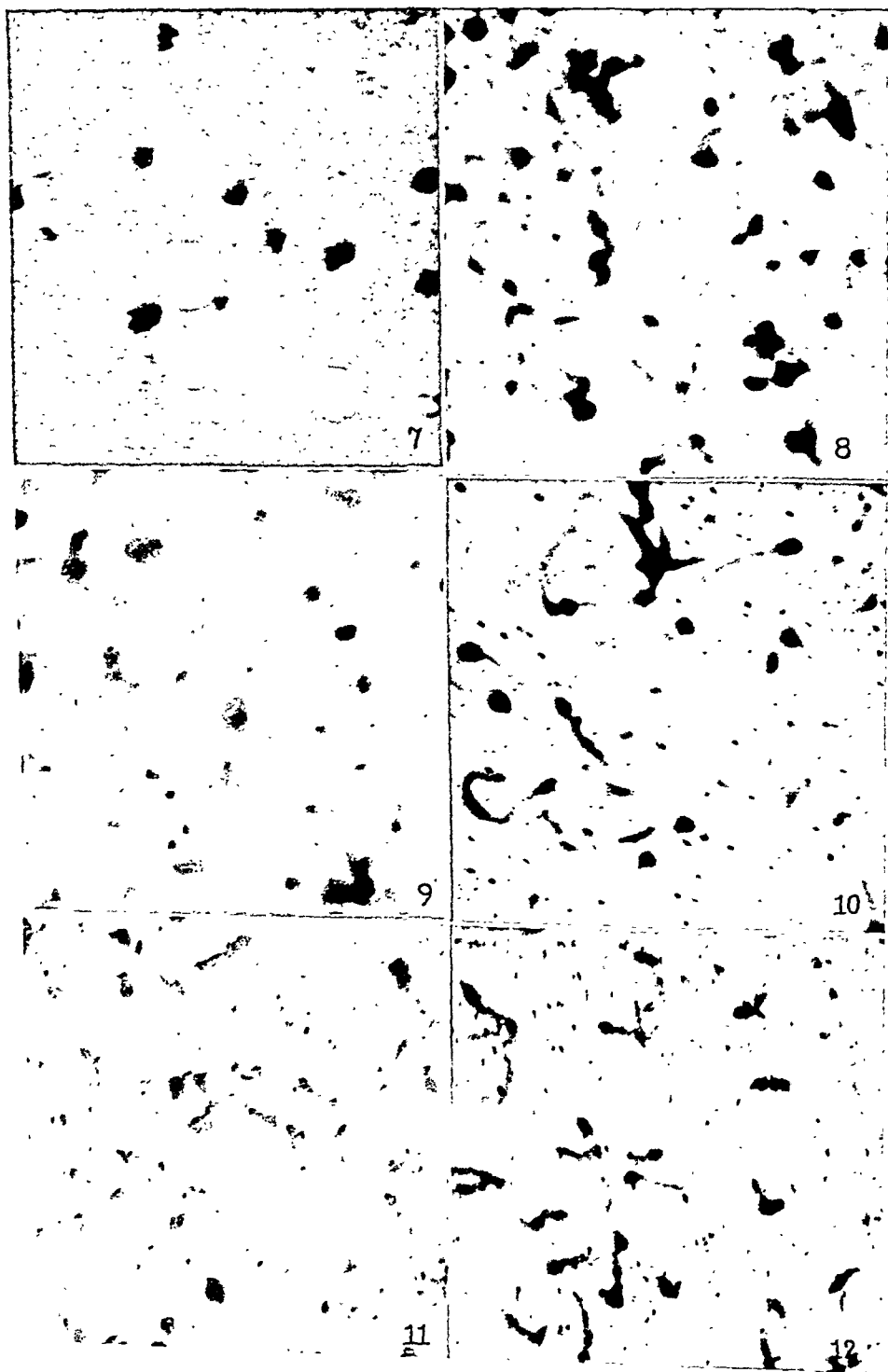
FIG. 8. Same at 0.05 M NaCl. × 18,000.

FIG. 9. Same at 0.10 M NaCl. × 18,000.

FIG. 10. Same at 0.15 M NaCl. × 18,000.

FIG. 11. Same at 0.20 M NaCl. × 18,000.

FIG. 12. Same at 0.25 M NaCl. × 18,000.



(Bang: Newcastle disease virus. III)

OBSERVATIONS ON THE FEVER CAUSED BY BACTERIAL
PYROGENSI. A STUDY OF THE RELATIONSHIP BETWEEN THE FEVER CAUSED BY
BACTERIAL PYROGENS AND THE FEVER ACCOMPANYING ACUTE
INFECTIONS*

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INTRODUCTION

Many bacteria produce substances capable of causing fever when injected into animals, and it seems possible that the fever accompanying acute infections may be attributable in some cases to a pyrogen produced by the infecting organism (1, 2). In the case of the fever following the intravenous injection of a pyrogenic substance such as typhoid vaccine, the time lag of 30 to 60 minutes before any rise in temperature suggests, as Beeson points out (3), that the pyrogen does not act directly on the hypothalamus. Since pyrogens are potent toxins, producing widespread tissue damage (4), it may be that their fever-promoting effect is secondary to injury.

The mechanism of the remarkable tolerance to the pyrogenic action of typhoid vaccine acquired by patients receiving multiple intravenous injections of this material for therapeutic purposes (5, 6) was studied by Beeson (3, 7). In 1947, he reported experiments demonstrating that rabbits acquire this tolerance readily and that an animal tolerant to one bacterial pyrogen is also insensitive in some degree to pyrogens produced by other organisms. The development of tolerance to a pyrogen was shown to be independent of the temperature rise following its injection, and mechanically induced fever failed to produce any tolerance. Beeson further showed that this tolerance is apparently dissociated from specific antibody formation and that the probable mechanism is an increase in the ability of the reticulo-endothelial system to remove the pyrogen from the circulation and thus prevent its temperature-raising effect. Tolerance is of short duration, being lost in 3 weeks.

If there exists any connection between the fever of acute infections and that produced by pyrogens of the infecting bacteria, animals convalescent from in-

* The opinions expressed in this report are to be construed as those of the author alone and do not reflect those of the Naval Medical Corps or the naval service at large.

fection might be expected to show tolerance for pyrogen. The present study was undertaken in an effort to define this relationship.

Though the amounts of pyrogen produced by bacteria of various types vary widely, it is generally true that the Gram-negative bacilli are the most potent producers of fever-promoting substances while the Gram-positive cocci are the least thermogenic (1). Therefore, the two infecting organisms chosen for this study were a Gram-negative bacillus and a Gram-positive coccus.

Materials and Methods

Animals.—Male New Zealand white rabbits weighing 2400 to 3100 gm. were used. They were caged in an air-conditioned room at 70° F. throughout the study. During tests involving

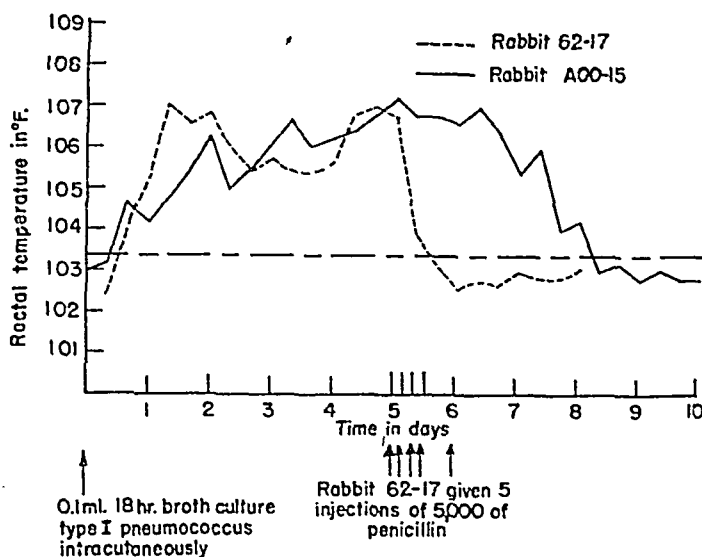


FIG. 1. Typical fever curve of an animal infected intradermally with Type I pneumococcus and allowed to recover without treatment, compared with fever curve of an animal with similar infection treated with penicillin on the 5th day of illness.

the injection of a pyrogen, they were placed in individual stalls and, after three rectal temperatures had been taken at 30 minute intervals to establish a normal level, the pyrogen was injected into the marginal ear veins. Rectal temperatures were then recorded every 30 minutes for 7 hours.

Infections.—Type I pneumococcus and *Escherichia coli* were used to produce infections in rabbits. The strain of pneumococcus was one obtained from the National Institute of Health and had been maintained in brain-heart infusion broth with weekly mouse passage. The intravenous injection of 9 billion of these organisms killed by heating at 60° C. for 30 minutes produced no significant temperature rise in rabbits. After the skin of the flank had been shaved, dermal pneumococcal infection of the type described by Goodner (8) was produced by the intracutaneous injection of 0.1 ml. of an 18 hour broth culture. The resulting infection was accompanied by high fever, and two-thirds of the infected animals died. In the surviving animals, fever ended by crisis on the 6th to 9th day. Ten animals were given 5,000 units of

penicillin intramuscularly every 4 hours on the 5th day of illness with 100 per cent recovery (Fig. 1).

Several strains of *E. coli* were tested for pathogenicity in rabbits with little success until a strain known to produce fatal peritonitis in mice was subjected to repeated passage in mice. Preliminary experimentation showed that the intraperitoneal injection of 1.0 ml. of an 8 hour broth culture of this organism in rabbits produced an illness accompanied by high fever with a duration of 7 to 8 days (Fig. 2). Approximately two-thirds of the animals thus infected survived.

Rectal temperatures of infected animals were recorded every 8 hours until a normal level (below 103.4° F.) was reached and maintained for at least 24 hours.

Pyrogenic Materials.—Two pyrogenic agents were used, *Salmonella typhi* and *E. coli* killed by heat. The dosages of these materials were adjusted so that each would cause marked pyrexia in rabbits without fatalities. The typhoid vaccine contained about 125 million *Sal-*

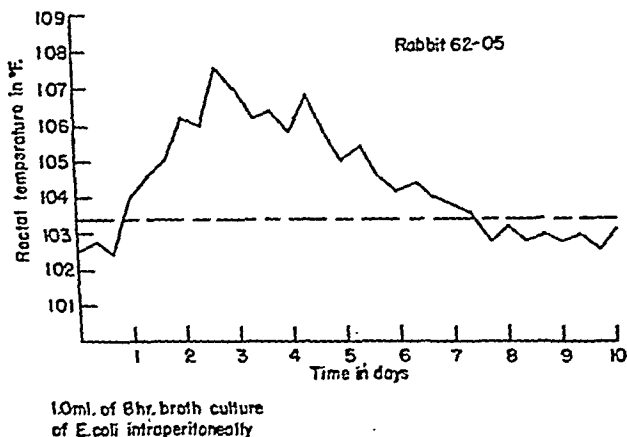


FIG. 2. Typical fever curve of an animal ill with peritonitis due to *E. coli*.

monella typhi organisms per ml. and was usually given in a dose of 1.0 ml. The comparable dose of *E. coli* was 300 million organisms in 1.0 ml. of physiologic saline.

All needles and glassware were sterilized in an oven at 170° C. for 3 hours to destroy any contaminating pyrogen. The physiologic saline used was tested frequently and was always pyrogen-free.

Method of Recording Results.—Following a method similar to that of Beeson, the temperature records after pyrogen injection were plotted on $\frac{3}{8}$ inch graph paper and, using the temperature at the time of injection as a base line, the area beneath the curve was measured with a Keuffel and Esser compensating planimeter, No. F4236. The vernier reading of the planimeter was taken as the "fever index," an expression of the height and duration of the fever.

RESULTS

Response to Single Injection of Pyrogen.—Forty-five animals received one injection of typhoid vaccine. The fever indices ranged from 92 to 193 with a mean of 128.4. Twenty-four animals were given one injection of *E. coli* vaccine. The fever indices of these animals varied from 98 to 200 with a mean

of 153.1. The first column in Fig. 3 shows the distribution of the fevers in these animals.

Effect of Repeated Injections of Pyrogens.—Twelve animals received daily injections of typhoid vaccine for 4 weeks. Beginning on the 4th day, a marked diminution in febrile response to the injections appeared. This reduction occurred both in height and duration of the fever. At the end of 4 weeks, each

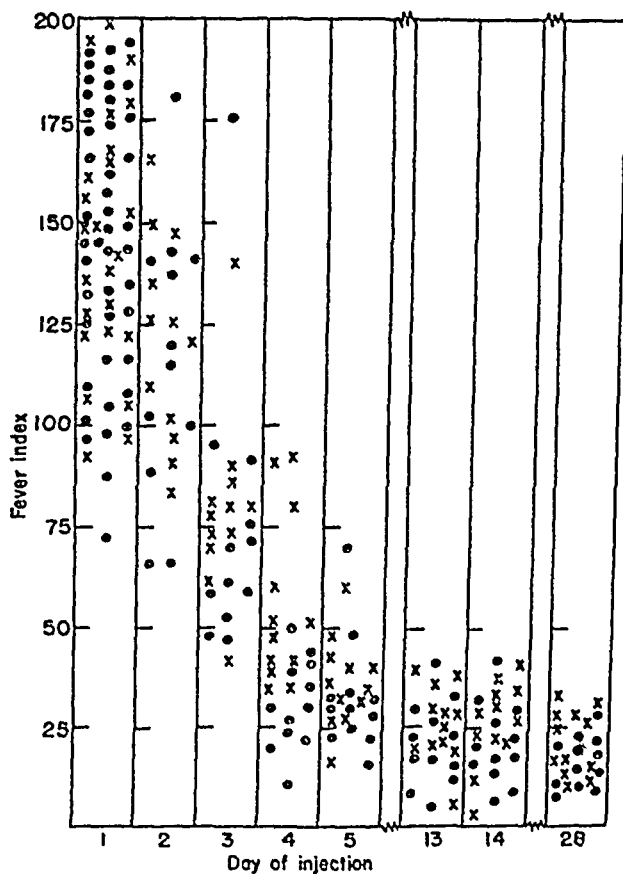


FIG. 3. Distribution of fevers recorded in animals at various times during the course of daily injections of 1.0 ml. of *S. typhi* (circles) or *E. coli* (crosses) vaccine.

of the animals tolerant to typhoid vaccine was given an injection of *E. coli* vaccine and the temperature response was recorded; they were also insensitive to the *E. coli* pyrogen, the fever indices for the group averaging 31.3. After a 3 week rest period, six animals received typhoid and six received *E. coli* vaccine; all tolerance had disappeared (Fig. 4).

Twelve animals were given daily injections of *E. coli* vaccine for 4 weeks. Again beginning on the 4th day, a marked lessening in the height and duration of the fever was apparent. At 4 weeks, these animals received typhoid

vaccine and exhibited tolerance for this material, their fever indices averaging 32.6. After a rest period of 3 weeks, six animals were given *E. coli*, and six, typhoid vaccine. All tolerance had disappeared during this interval (Fig. 5).

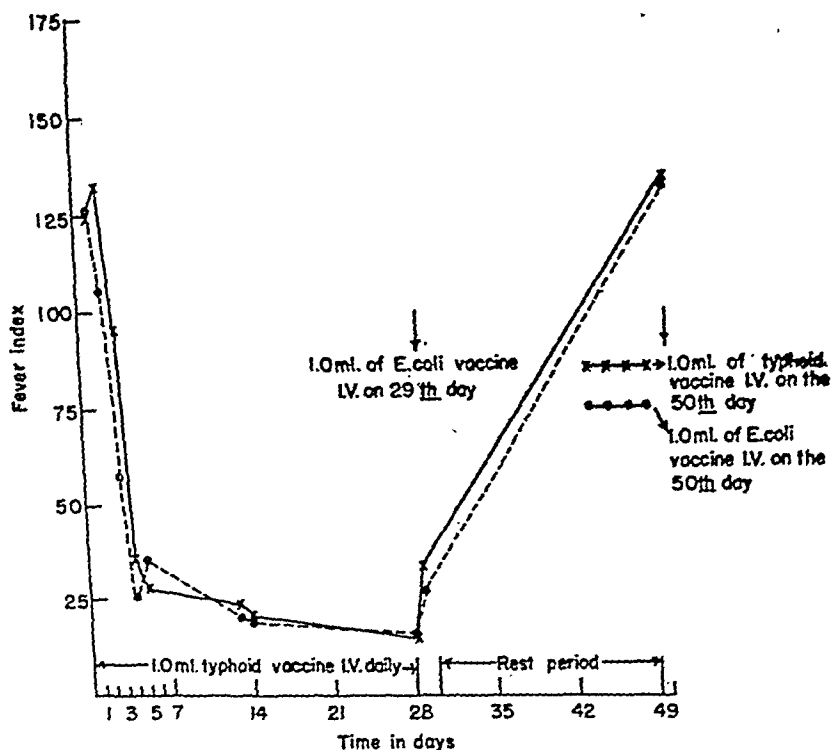


FIG. 4. Average fever indices in two groups of six rabbits given 1.0 ml. of *S. typhi* vaccine daily for 4 weeks. Note that by the 4th day the febrile responses had dropped almost to the level reached at the 28th day. On the 29th day, both groups received 1.0 ml. of *E. coli* vaccine, with only slight increase in the fever indices, indicating their tolerance for this pyrogen as well. The increased response to both vaccines after a rest period of 3 weeks indicates loss of tolerance with discontinuance of daily injections.

This study was repeated, employing smaller doses of pyrogen. One ml. of the 1:10 dilution of typhoid vaccine contained 12.5 million bacilli; the *E. coli* vaccine was also used in a dose of 1.0 ml. of a 1:10 dilution, or 30 million bacilli. With repeated injections, animals developed tolerance to these smaller doses (Fig. 6).

Effect of Pneumococcal Infection upon Response to Pyrogens.—Ten animals surviving infection with Type I pneumococcus received 1.0 ml. of typhoid vaccine 24 hours after their temperatures had returned to normal, and a second

injection 3 weeks after infection since any tolerance resulting from infection would have disappeared by this time. Five of the animals in this group had recovered from the infection without treatment and five had received penicillin on the 5th day. There was no evidence of any tolerance to typhoid vaccine in

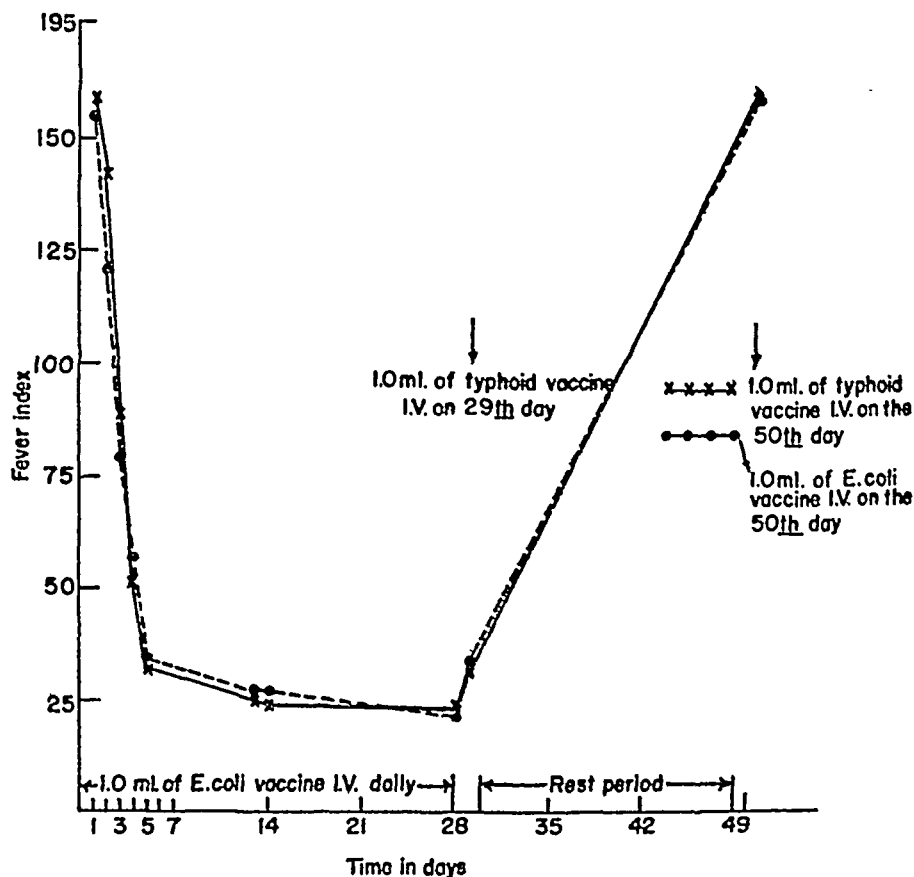


FIG. 5. Average fever indices of two groups of six animals showing development of tolerance to daily injections of *E. coli* vaccine over a period of 4 weeks, with slight rise in fever indices when *S. typhi* vaccine was substituted on the 29th day. Again the high fevers in response to both vaccines after 3 weeks indicate the disappearance of pyrogen tolerance.

any of these animals, the indices averaging 154.1 at 24 hours and 153.5 at 3 weeks (Table I).

A second group of animals surviving dermal pneumococcal infection showed no tolerance to 1.0 ml. of *E. coli* vaccine. The fever indices averaged 133.4 at 24 hours and 140.3 at 3 weeks (Table II).

Effect of E. coli Infection upon Response to Pyrogens.—Twelve animals surviving *E. coli* peritonitis were given 1.0 ml. of typhoid vaccine 24 hours and again 3 weeks after their temperatures had returned to normal. There was no

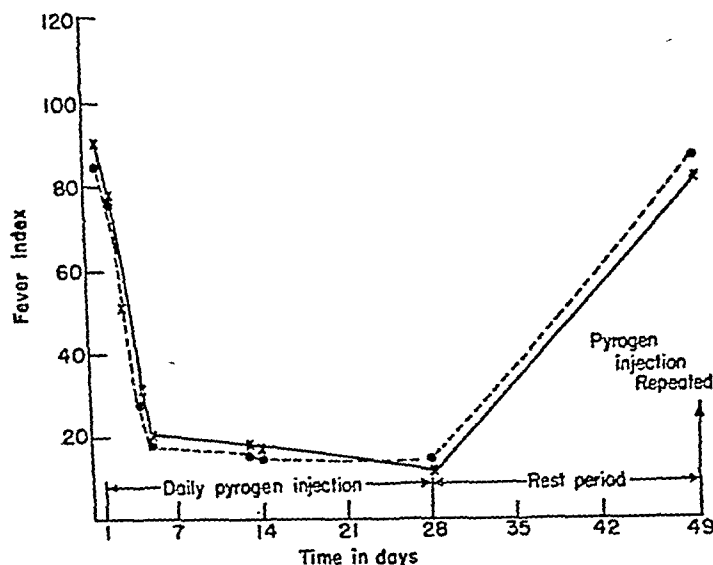


FIG. 6. Average fever indices of two groups of six animals, one receiving 0.1 ml. of *E. coli* vaccine daily for 4 weeks and the other a similar dose of *S. typhi* vaccine. Note the development of tolerance to these smaller amounts of pyrogen with loss of tolerance after discontinuance of injections for 3 weeks.

TABLE I

Response of Ten Animals, Convalescent from Dermal Infection with Type I Pneumococcus, to Injection of 1.0 Ml. of Typhoid Vaccine 24 Hours and 3 Weeks after Return of Temperatures to Normal

Animal No.	Penicillin on 5th day	Duration of infection	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
		days		
90-27	Yes	6	168	156
90-38	Yes	6	134	153
90-47	Yes	6	213	178
90-40	Yes	6	126	133
65-26	Yes	6	128	112
62-16	No	8	140	135
A0-01	No	7	145	152
A0-02	No	8	157	163
A0-04	No	8	179	180
A0-06	No	7	151	173
Average.....			154.1	153.5

evidence that the infection had produced any tolerance to the *S. typhi* pyrogen, the fever indices averaging 161.3 at 24 hours and 162.5 at 3 weeks. This

experiment was repeated with twelve animals, using *E. coli* vaccine instead of typhoid. The fever indices, which averaged 166.6 at 24 hours and 168.0 at 3

TABLE II

Response of Ten Animals, Convalescent from Dermal Pneumococcal Infection, to Intravenous Injection of 1.0 Ml. of E. coli Vaccine 24 Hours and 3 Weeks after Return of Temperatures to Normal

Animal No.	Penicillin on 5th day	Duration of infection	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
		days		
A2-32	Yes	6	98	130
A2-33	Yes	6	127	140
A2-34	Yes	6	171	154
A2-35	Yes	6	163	160
A2-36	Yes	6	128	144
A2-23	No	6	113	142
A2-25	No	8	122	138
A2-29	No	8	104	109
A2-30	No	7	161	147
A2-31	No	8	147	139
Average.....			133.4	140.3

TABLE III

Response of Twelve Animals to Injection of 1.0 Ml. of Typhoid Vaccine 24 Hours and 3 Weeks after Recovery from E. coli Peritonitis

Animal No.	Duration of infection	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
	days		
45-51	7	211	194
45-52	8	114	143
45-54	9	168	198
45-55	7	127	136
45-58	7	156	163
45-59	9	147	151
45-60	9	171	160
45-61	8	187	172
45-62	9	193	147
45-66	7	138	156
45-67	9	171	142
45-68	8	153	188
Average.....		161.3	162.5

weeks, demonstrated that active infection with the colon bacillus had produced no tolerance to the fever-promoting effect of this dose of pyrogen derived from the same strain (Tables III and IV).

Smaller doses of pyrogen were given to animals surviving *E. coli* peritonitis. Six convalescent animals received 1.0 ml. of the 1:10 dilution of typhoid vaccine

TABLE IV

Response of Twelve Animals to Injection of 1.0 Ml. of E. coli Vaccine 24 Hours and 3 Weeks after Recovery from E. coli Peritonitis

Animal No.	Duration of infection	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
	days		
45-71	8	197	143
45-73	9	156	203
45-77	7	188	181
45-78	8	173	184
45-79	8	162	190
45-81	9	141	128
45-82	9	157	151
45-83	7	168	172
45-86	8	214	192
45-87	8	162	198
45-88	9	130	136
45-89	7	151	138
Average		166.6	168.0

TABLE V

Response of Twelve Animals to Injections of 0.1 Ml. of Typhoid or E. coli Vaccine 24 Hours and 3 Weeks after Recovery from E. coli Peritonitis

Animal No.	Duration of infection	Pyrogenic material used	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
	days			
90-35	8	<i>S. typhi</i>	81	76
90-36	9	" "	93	74
90-37	7	" "	88	94
90-38	8	" "	109	108
90-39	8	" "	76	83
90-40	8	" "	104	94
90-44	9	<i>E. coli</i>	110	98
90-45	9	" "	91	90
90-46	7	" "	69	79
90-47	7	" "	78	73
90-48	9	" "	87	106
90-49	8	" "	93	88
Average.....			89.0	88.6

at 24 hours and 3 weeks and six animals received 1.0 ml. of the 1:10 dilution of *E. coli* vaccine. There was no evidence that the infection had produced tolerance for these smaller amounts of pyrogen (Table V).

Effect of Concomitant Infection upon Development of Tolerance to Repeated Injections of Pyrogens.—Twelve rabbits with *E. coli* peritonitis received daily injections of 1.0 ml. of the 1:10 dilution of typhoid vaccine throughout the course of the infection. It was necessary to use the smaller dose of pyrogen in

TABLE VI

Response of Seven Animals, Given Daily Injections of 0.1 Ml. of Typhoid Vaccine during the Course of E. coli Peritonitis, to a Repeated Dose of Vaccine 24 Hours and Again 3 Weeks after Recovery from Infection

Animal No.	Duration of infection	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
	days		
91-26	8	21	81
91-28	9	32	106
91-29	7	17	92
91-33	6	19	73
91-34	9	27	112
91-36	8	17	68
91-37	9	28	99
Average		23.0	90.1

TABLE VII

Response of Five Animals Given Daily Injections of 0.1 Ml. of E. coli Vaccine during the Course of E. coli Peritonitis, to a Repeated Dose of Vaccine 24 Hours and Again 3 Weeks after Recovery from Infection

Animal No.	Duration of infection	Fever index 24 hrs. after recovery	Fever index 3 wks. after recovery
	days		
91-40	9	25	84
91-44	8	31	78
91-48	7	11	91
91-49	9	27	77
91-51	8	24	82
Average		23.6	82.4

these studies in order to obtain survivors since the combination of the peritonitis and the larger doses almost invariably caused hyperpyrexia and death. The temperatures of the seven surviving animals had returned to normal in 7 or 8 days. 24 hours and again 3 weeks after recovery, the injection of typhoid vaccine was repeated (Table VI). The fever indices at 24 hours were low (average, 23.0), indicating that the animals were capable of developing tolerance to pyrogen in the presence of infection. This tolerance had disappeared in 3 weeks (average, 90.1). In a similar experiment using *E. coli* vaccine, the fever

indices of the five surviving animals averaged 23.6 at 24 hours and 82.4 at 3 weeks, indicating the development of tolerance to the *E. coli* pyrogen during the infection, and its subsequent loss (Table VII).

DISCUSSION

The results presented above confirm the finding of Beeson that rabbits will become tolerant to bacterial pyrogens if given a course of daily injections. Animals tolerant to one pyrogen are also insensitive in large measure to pyrogens produced by other organisms. The duration of the tolerance is less than 3 weeks.

Although fever accompanying various types of disease is attributable in a general way to some imbalance of the temperature-regulating centers, the mechanism involved is poorly understood. In cases of brain trauma or increased intracranial pressure, mechanical stimulation undoubtedly plays a part. Whether there is a common factor underlying the fever of such varied conditions as acute infections, hemolytic crises, serum sickness, rheumatic fever, pernicious anemia, neoplasms, hepatic failure, trauma, and myocardial infarction is not definitely known. The presence of tissue injury is a characteristic of all these states and it seems possible that some product of cell damage or altered cell metabolism may act on the hypothalamus to produce fever accompanying them. Menkin (9) has isolated from inflammatory exudates a substance which he considers responsible for the fever accompanying acute inflammatory states. This material he terms *pyrexin* and describes as a product of cell injury. His work has not been confirmed up to this time.

The failure of animals infected with Type I pneumococcus to show tolerance for pyrogens after recovery is not surprising, since this organism produces little or no pyrogen demonstrable by the methods used in this study. The failure of actual febrile infection with the colon bacillus to produce tolerance for pyrogens in rabbits seemed to indicate that the pyrogen produced by this organism plays little or no part in the production of the fever accompanying infection or that the conditions necessary for the development of this tolerance are not present during infection (perhaps due to some effect on the reticulo-endothelial system). Animals receiving repeated pyrogen injections during infection, however, developed tolerance in the usual fashion. Thus, it seems probable that the pyrogen produced by *E. coli* is not the primary factor responsible for the production of fever accompanying infections with this organism.

According to the hypothesis that fever of infection is caused by some product of cellular injury, the failure of *E. coli* infection to confer tolerance to the fever-promoting effect of its pyrogen may be explained by assuming that the pyrogen itself is not the primary cause of tissue injury in infection, though the introduction of a comparatively large quantity of pyrogen directly into the blood stream brings about enough cell injury to cause fever.

These findings, then, apparently represent another example of the difficulty frequently experienced in correlating the manifestations of disease with properties of specific toxic fractions of the causative organism.

SUMMARY AND CONCLUSIONS

The relationship between the fever of acute infection and that following injection of bacterial pyrogen was studied by administering pyrogens to animals convalescent from acute infections.

Rabbits surviving dermal pneumococcal infections or peritonitis due to *Escherichia coli* were given intravenous injections of typhoid or *E. coli* vaccine. They showed no evidence of tolerance to the fever-promoting effect of these pyrogenic materials.

Tolerance did develop in infected animals given daily pyrogen injections during the course of the infection.

Certain previous observations upon the ability of rabbits to develop tolerance to pyrogens, the broad nature of the tolerance, and its duration were confirmed.

It is concluded that the pyrogen produced by certain bacteria plays little or no rôle in the production of the fever of infection.

These findings are compatible with the hypothesis that there is a common factor, perhaps a product of cell injury, underlying the fever accompanying diseases of various types.

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OBSERVATIONS ON THE FEVER CAUSED BY BACTERIAL PYROGENS

II. A STUDY OF THE RELATIONSHIP BETWEEN THE FEVERS CAUSED BY BACTERIAL PYROGENS AND BY THE INTRAVENOUS INJECTION OF THE STERILE EXUDATES OF ACUTE INFLAMMATION*

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INTRODUCTION

Menkin (1) has isolated from sterile exudates in dogs a material closely associated with the euglobulin fraction which causes a febrile response when injected intravenously in rabbits. This substance he described as a product of cell injury and termed *pyrexin*. He attributed the fever accompanying acute inflammatory states to the action of pyrexin produced at the site of inflammation. Menkin's work is open to the criticism that he failed to mention precautions against the ever present danger of contamination with bacterial pyrogens during the process of fractionating the exudate. The sterility of the whole exudate and the fractions derived from it is only one indication of freedom from such contamination, since pyrogens are heat-stable and can cause fever in extremely small quantities (2, 3).

If the fever-promoting effect of sterile exudates is due to the presence of bacterial pyrogen, animals receiving a course of daily injections of an exudate should develop tolerance for this effect and should also show comparative insensitivity to the effects of other pyrogens (4, 5). The present study was undertaken in an attempt to define the relationship between the fever following injection of exudates in rabbits and that caused by bacterial pyrogens.

Materials and Methods

Sterile exudates were produced in dogs by the intrapleural injection of 1.5 to 2.0 ml. of turpentine as described by Menkin (6). Following the injection of this irritant, daily thoracenteses were performed and about 15 ml. of chest fluid was withdrawn for examination. Clotting was prevented by the addition of heparin. Cultures were made immediately in thioglycollate broth, the pH was tested, and smears were examined for bacteria and differential cell count. The cultures were examined after 24 and 48 hours of incubation and any exudate

* The opinions expressed in this report are to be construed as those of the author alone and do not reflect those of the Naval Medical Corps or the naval service at large.

showing evidence of bacterial contamination was discarded. The pyrogenicity of the whole exudate was tested each day by intravenous injection in rabbits.

As Menkin described (1, 6), with progression of the inflammatory process, the reaction of the exudate, in most cases, changed from alkaline to acid within 4 to 6 days. The cellular content consisted almost entirely of polymorphonuclear leucocytes at first but this gradually gave way to a predominance of mononuclear cells by the time that the fluid had become acid.

Within 24 to 48 hours after the exudates became acid, they usually caused definite febrile responses in rabbits. However, three of nine acid exudates failed to produce fever and, on one occasion, an exudate with an alkaline reaction proved to be pyrogenic.

Rectal temperatures and circulating leucocyte counts were recorded twice daily in dogs injected with turpentine. These followed a fairly consistent pattern (Fig. 1). Fever generally

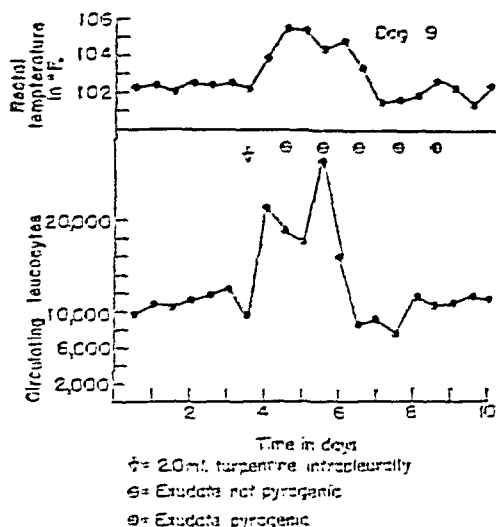


FIG. 1. Rectal temperature and circulating leucocytes in a dog given turpentine intrapleurally on the 4th day. Note that the dog's temperature had returned to normal by the time that the exudate had become pyrogenic. The sudden drop in leucocytes on the 7th day, 24 hours before the appearance of the fever-promoting factor in the chest fluid, is characteristic.

lasted about 3 days. By the time the fever-promoting factor could be demonstrated in the chest fluid, the dog's temperature had returned to normal. The circulating leucocytes increased in number after administration of turpentine and this increase persisted for 3 or 4 days, after which there was a sudden sharp drop about 24 hours before the exudate became pyrogenic. This sudden fall in leucocyte count almost invariably preceded the appearance of the fever-promoting factor in the chest fluid and came to be recognized as a reliable sign of its presence.

When an exudate had been shown to be pyrogenic and cultures were negative, thoracentesis was performed and as much of the fluid as could be withdrawn was collected in sterile, pyrogen-free glass containers and stored at 4°C. Over periods as long as 2 months, no diminution was observed in the fever-promoting property of fluids so stored. No attempt was made to separate various components chemically.

Temperatures of rabbits following injection of exudates or bacterial pyrogens were recorded every 30 minutes for 7 hours and a "fever index" (5) computed to facilitate comparison of responses. The bacterial pyrogens employed were typhoid and *Escherichia coli* vaccines.

RESULTS

Comparison of Fever Curves Produced by Injection of Exudate and by Injection of Bacterial Pyrogen.—The febrile response in rabbits following the injection of chest fluid differed from that following the injection of a bacterial pyrogen in that after a brisk rise to a peak at $1\frac{1}{2}$ to 2 hours, there was an abrupt fall of the temperature to the initial level within $3\frac{1}{2}$ hours. Though larger doses of the exudate could raise the peak of the fever, the duration of the elevation remained the same. With bacterial pyrogen, the response is prolonged, the

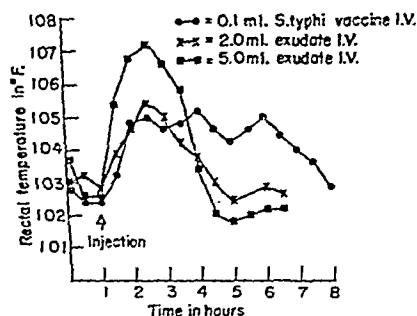


FIG. 2

FIG. 2. Mean fever curves obtained in a group of four rabbits after administration on successive days of 0.1 ml. of *S. typhi* vaccine, 2.0 ml. of exudate, and 5.0 ml. of exudate. Note the rapid rise and fall after injections of exudate as compared to the prolonged response after the bacterial pyrogen.

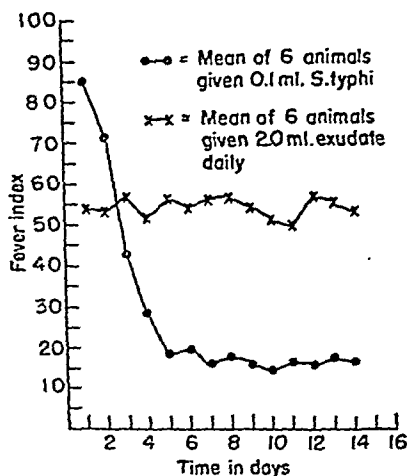


FIG. 3

FIG. 3. Comparison of the response of six animals to daily injections of exudate with the progressive diminution in response of six animals given daily injections of bacterial pyrogen.

temperature sometimes failing to return to the base line within the observation period of 7 hours (Fig. 2).

Effects of Repeated Injections of Exudates in Normal Animals.—Three groups of rabbits received daily injections of 2.0 ml. of whole exudate for from 10 to 21 days. In sharp contrast to the diminishing response to daily injections of bacterial pyrogens, there was no evidence that tolerance to the fever-promoting property of the exudates developed in these animals (Fig. 3).

Effect of Exudates in Animals Tolerant to Bacterial Pyrogens.—Six animals rendered tolerant by daily injections of 0.1 ml. of typhoid vaccine for 14 days were given 3.0 ml. of exudate intravenously on the 15th day. In normal animals, the fever index following injection of this amount of exudate ap-

proximated that resulting from 0.1 ml. of this vaccine. There was no evidence of tolerance to the exudate, since all animals responded with brisk fevers.

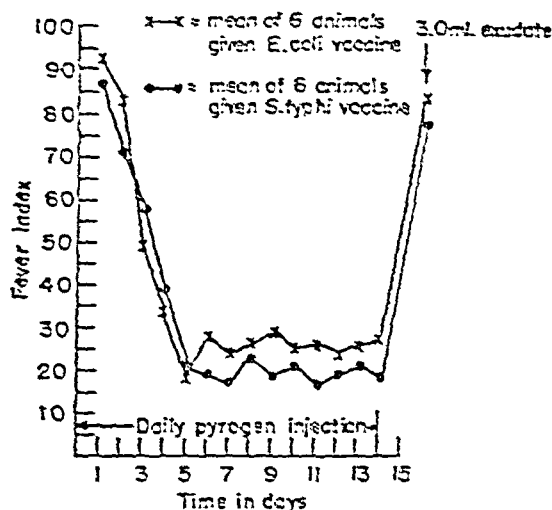


FIG. 4. Failure of two groups of animals rendered tolerant to bacterial pyrogens by daily injections for 14 days to show tolerance for the fever-promoting action of exudate given on the 15th day.

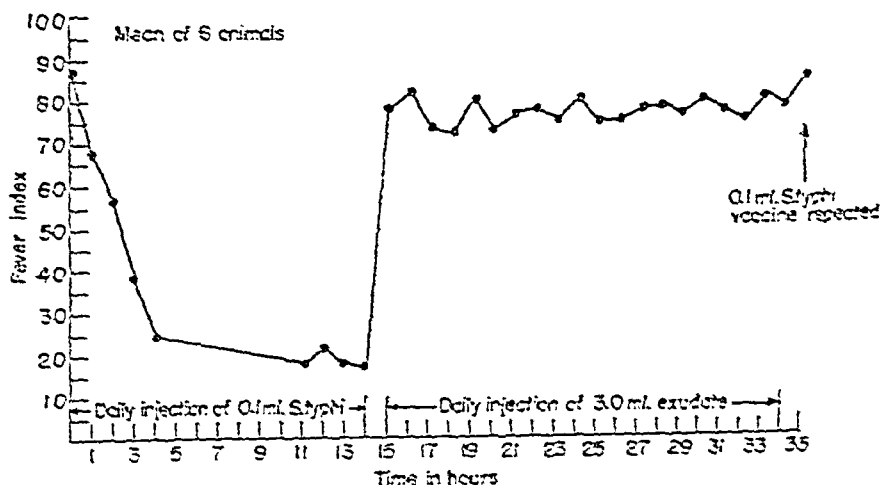


FIG. 5. Chart showing febrile responses of six animals given daily injections of bacterial pyrogen until tolerance appeared, then given daily injections of exudate for 3 weeks. At the end of this time, the high fever, after a repeated injection of bacterial pyrogen, indicated that the daily injections of exudate failed to maintain tolerance in these animals. Tolerance for bacterial pyrogens is normally lost in about 3 weeks.

This experiment was repeated with animals made tolerant to *E. coli* vaccine and again no tolerance to the temperature-raising effect of the exudate was noted (Fig. 4).

Finally, a group of animals tolerant to typhoid vaccine received intravenous injections of 3.0 ml. of exudate daily for 21 days. Tolerance to bacterial pyrogens normally lasts about 3 weeks (4, 5). At the end of this time, a repeated injection of typhoid vaccine was followed by high fever, indicating that tolerance for this bacterial pyrogen had not been maintained by daily injections of the exudate (Fig. 5).

DISCUSSION

These findings confirm Menkin's observation that there is present in the chest fluid of dogs given an intrapleural injection of turpentine a substance which causes definite febrile response in rabbits.

The failure of rabbits to develop tolerance to repeated injections of sterile exudates, the failure of animals tolerant to bacterial pyrogens to show tolerance to the fever-promoting effect of these exudates, and the failure of repeated injections of exudate to maintain tolerance for bacterial pyrogen, indicate that the production of fever by these exudates is not due to contamination with bacterial pyrogen. The shorter duration of the febrile response following the injection of exudate as compared with that following administration of bacterial pyrogens furnishes additional evidence that the substance in the exudate which causes fever is not a bacterial product.

The fact that the exudate itself is not pyrogenic until the dog's temperature has returned to normal makes it difficult to attribute the dog's fever to absorption of this substance.¹ Further studies of this apparent inconsistency are under way. The significance of the sudden drop in circulating leucocyte count shortly before the appearance of the fever-promoting factor in the chest fluid is also under investigation.

SUMMARY AND CONCLUSIONS

The relationship of the fever caused in rabbits by bacterial pyrogens to the fever produced by the injection of the sterile exudates of acute inflammation was investigated by recording the responses of normal and pyrogen-tolerant animals to injections of exudate. Exudates were produced by the intrapleural injection of turpentine in dogs.

The duration of the febrile response in rabbits after a single dose of exudate was found to be much shorter than the fever following an injection of bacterial pyrogen.

Animals given daily injections of exudate demonstrated no tolerance to its fever-promoting effect.

Animals tolerant to bacterial pyrogens showed no diminution in responsiveness to exudates.

¹ The intravenous injection of chest fluid in dogs also causes a febrile response.

Daily injections of exudate failed to establish tolerance to bacterial pyrogens in rabbits.

It is concluded that the fever-promoting property of sterile exudates is not due to the presence of bacterial pyrogen.

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STUDIES ON THE RELATIONSHIP OF PTEROYLGLUTAMIC ACID TO THE GROWTH OF PSITTACOSIS VIRUS (STRAIN 6BC)*

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The studies by Woods (1) on the relationship of *p*-aminobenzoic acid (PABA) to the growth inhibitory action of the sulfonamides for bacteria have provided a useful tool for metabolic studies with PABA and related compounds such as pteric acid and pteroylglutamic (synthetic folic) acid (PGA) which contain the PABA moiety (2, 3). Demonstration of the growth inhibitory effect of sulfadiazine on psittacosis virus (strain 6BC) (4) and the subsequent studies showing that this inhibitory action was antagonized by PABA and PGA (5) suggested the usefulness of this system for a study of certain factors concerned in the growth of this virus. The present communication reports the results of experiments on the effect of PABA, pteric acid, PGA, and related compounds on sulfonamide inhibition of the growth of psittacosis virus and attempts to use this phenomenon to study certain possible synthetic activities of this virus.

Since certain analogues of PGA¹ have recently become available, it was of interest to test them for their effect on growth of the virus and for their influence on the inhibitory action of the sulfonamides since, in other systems, some of them behave like PGA (2) and others act as PGA antagonists (6, 7).

Materials and Methods

Psittacosis virus (strain 6BC) which had been repeatedly passed in eggs by the yolk sac route was used. A pool of infected yolk sacs was prepared in nutrient broth with a Waring blender. Aliquots were placed in sealed glass ampoules and stored in the dry ice cabinet to provide a uniform inoculum for use in the experiments. This yolk sac suspension was titrated by injecting 0.25 ml. amounts of serial tenfold dilutions into the yolk sac of 6 to 7 day old embryonated eggs. The infected eggs were incubated at 35°C. for 10 days during which time deaths were recorded and the LD₅₀ titer calculated (8). Deaths occurring during the first 48 hours were considered traumatic and disregarded. The day of death of the embryos showed an inverse correlation with the size of the infectious inoculum. For subsequent experiments, a dilution of the seed virus containing 10,000 LD₅₀ doses in 0.25 ml. was used.

The various compounds to be tested were dissolved in sterile distilled water for injection. The sulfadiazine was obtained as a sterile solution of the sodium salt (NaSD) and dosage was

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† Senior Fellow in Medical Sciences of the National Research Council.

¹ Obtained through the courtesy of Lederle Laboratories.

allantoic fluids after 2, 4, 6, and 9 days. With doses of PGA as large as 10 mg. per egg, the yolk and allantoic fluids contained no free PABA after the same

TABLE I

Determination of the Minimal Inhibiting Dose of NaSD on the Growth of Psittacosis Virus (Strain 6BC) in Eggs

NaSD	No. of eggs	Survived 10 days*
mg.		per cent
5.0	24	96
2.5	29	97
1.25	29	90
0.5	16	82
0.1	25	0
0.05	12	0
0.01	22	0.
0	8	0

* 10,000 LD₅₀ virus injected *via* yolk sac.

TABLE II

Effect of PABA and PGA on the Growth Inhibition of Psittacosis Virus (Strain 6BC) by NaSD

NaSD	Inhibitor	No. of eggs	Survived 10 days*
mg.	mg.		per cent
2.5	PABA 0.5	12	0
"	" 0.05	11	0
"	" 0.005	12	0
"	" 0.001	9	55
"	" 0.0005	10	100
"	PGA 5.0	12	0
"	" 0.5	12	0
"	" 0.25	11	66
"	" 0.01	10	100
"	" 0.005	9	100
"	— 0	12	92
0	PABA 5.0	8	0
0	PGA 10.0	10	0
<i>Drug Controls†</i>			
0	PABA 5.0	20	95
0	PGA 10.0	19	95

* 10,000 LD₅₀ virus injected *via* yolk sac.

† Not infected.

intervals of time. This evidence strongly suggests that PGA is not broken down with release of PABA to any appreciable extent and, therefore, that PGA

PABA and 5 mg. of PGA these compounds failed to show any effect on the protective action of 500 units of penicillin which gave an 80 per cent survival rate in embryos infected with 10,000 LD₅₀ of psittacosis virus. This provides additional evidence for the specific nature of the metabolic interrelationships of NaSD, PABA, and PGA in the growth of psittacosis virus.

Since liver extract as well as PGA has an important relationship to pernicious anemia, liver extract was tested in doses of 4.2 units (purified, Lilly) for possible antagonism of 2.5 mg. NaSD and was shown to be without any effect on the action of the drug.

Type of Sulfonamide Antagonism Produced by PABA and PGA

The previous studies of sulfonamide antagonism with bacteria had shown that PABA exerted a competitive antagonism on the sulfonamides, *i.e.* the amount of antagonist required bore a direct relationship to the dose of sulfonamide used, while PGA exerted its effect without regard to the amount of sulfonamide present (2, 3). It was of interest, therefore, to determine the nature of the action of these antagonists in the system being studied. Two doses of PABA and PGA, representing 2 and 10 times their minimal effective amounts for 2.5 mg. of NaSD, were therefore tested against increasing amounts of NaSD. These data are presented in Table III.

There is a direct relationship between the amount of SD used and the amount of PABA required to antagonize its inhibitory effect which demonstrates a competitive type of inhibition for PABA. On the other hand, once an effective dose of PGA is given, the dose of NaSD may be increased as much as 50 times without overcoming this antagonistic effect. Therefore, PGA exerts a non-competitive type of antagonism.

Effect of Other Components of PGA on Sulfonamide Action

Since PGA is composed of glutamic acid, PABA, and the pteridine nucleus, it was of interest to test components other than PABA for sulfonamide antagonism. Glutamic acid and pteric acid were available for testing. The pteric acid had been purified so that it contained only 0.026 per cent of free PABA and 7 per cent of other pterin materials as contaminants.² Table IV presents the data obtained in these experiments.

Results of these experiments show that glutamic acid in doses up to 10 mg. exerts no effect on the sulfonamide inhibition of virus growth while pteric acid in 0.05 and 0.25 mg. amounts shows a competitive antagonistic effect.

Effects of Certain Analogues of PGA on the Growth of Psittacosis Virus and Its Inhibition by NaSD

The following analogues of PGA were tested: pteroyldiglutamic acid, pteroyl- γ -diglutamic acid, pteroyltriglutamic acid, pteroylaspartic acid, and 4-amino-

² Data furnished by Dr. C. W. Waller of the Lederle Laboratories.

amounts of PABA present in this preparation. It had no effect on the growth of the virus in 10 mg. amounts.

Attempts to study the effect of 4-aminopteroylglutamic acid on the growth of the virus were difficult since this compound produced deaths in from 5 to 10

TABLE V

Effect of Analogues of Folic Acid on the Growth of Psittacosis Virus and Its Inhibition by NaSD

NaSD	Inhibitor		No. of eggs	Survived 10 days*
mg.	mg.			per cent
2.5	Pteroylglutamic acid	0.1	8	0
"	" "	0.5	10	0
"	Pteroyldiglutamic acid	0.1	10	10
"	" "	0.5	9	0
"	Pteroyl- γ -diglutamic acid	0.1	10	0
"	" "	0.5	10	0
"	Pteroyltriglutamic acid	0.1	8	0
"	" "	0.5	10	0
"	Pteroylaspartic acid	0.1	10	50
"	" "	0.5	10	0
"	N-methylpterioic acid	0.5	6	66
"	" "	5.0	8	0
0	Pteroyldiglutamic acid	2.5	8	0
0	Pteroyl- γ -diglutamic acid	2.5	8	0
0	Pteroyltriglutamic acid	2.5	8	0
0	Pteroylaspartic acid	10.0	6	0
0	N-methylpterioic acid	10.0	7	0
2.5	—	0	12	100
0	—	0	12	0
Drug controls†				
0	Pteroyldiglutamic acid	2.5	10	100
0	Pteroyl- γ -diglutamic acid	2.5	10	100
0	Pteroyltriglutamic acid	2.5	10	90
0	Pteroylaspartic acid	10.0	9	75
0	N-methylpterioic acid	10.0	10	100

* 10,000 LD₅₀ virus injected *via* yolk sac.

† Not infected.

days in chick embryos when given in doses of 0.001 to 0.005 mg. When virus-infected eggs were given 3 injections of 0.002 mg. over a period of 7 days and the yolk sacs were harvested and titrated, the content of virus was about the same as in untreated and infected controls. Small doses of 4-aminopteroylglutamic acid (0.002 mg.) combined with 0.25 to 0.01 mg. of NaSD gave no evidence of potentiating nor antagonizing the virus-inhibiting action of the

LEPTOSPIROSIS IN CATTLE*

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PLATES 10 TO 12

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During a study of bovine mastitis in groups of Holstein, Guernsey, and Brown Swiss dairy cows in April, 1946, three animals were observed whose milk had a bloody or thickened yellowish appearance. The incidence of this condition increased markedly in May and occasional cases have continued to occur. The affected cows showed fever and rarely hemoglobinuria; a few showed inappetence and a lowered milk yield.

The clinical manifestations and incidence indicated an infectious disease, and since bacteriological examinations of the milk revealed a mixed bacterial flora of no apparent etiological significance, attempts were made to transmit an infectious agent to laboratory animals.

Transmission Studies in Laboratory Animals

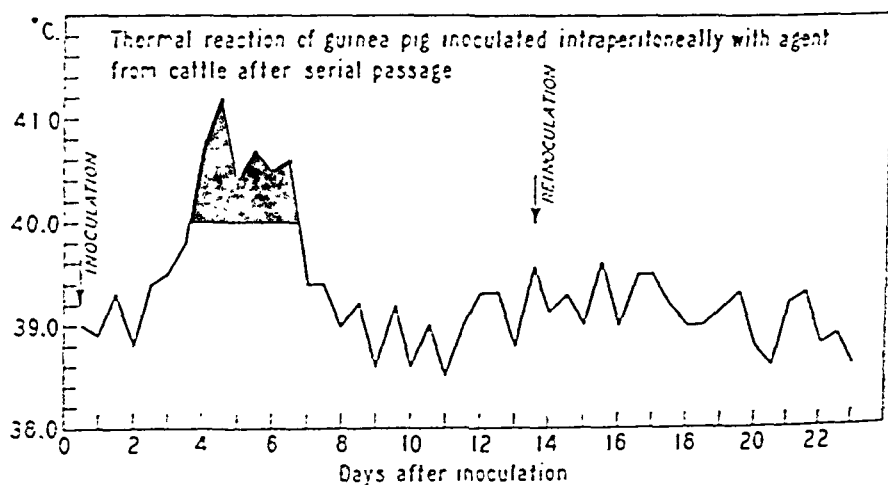
Guinea Pigs.—A stock of apparently healthy guinea pigs was moved into a constant temperature room which was maintained at 70°F. with 60 per cent humidity. Normal animals kept under these conditions never showed rectal temperatures exceeding 40°C. and readings above this were interpreted as fever. Groups of 2 or 3 guinea pigs weighing 250 to 350 gm. were each inoculated intraperitoneally with 1 to 5 cc. of freshly drawn abnormal milk. Thereafter rectal temperatures were taken twice daily on all animals and daily weighings made on selected groups. Defibrinated blood, obtained by heart puncture from guinea pigs showing fever, was inoculated into additional guinea pigs, as had been done for the original passage, with the exception that the size of animals varied from 150 to 600 gm.

A pathogenic agent was recovered from each of 5 specimens of abnormal milk, 3 of which were taken at the height of the outbreak (C161 on May 27, 1946, C162 on June 19, 1946, and C164 on June 19, 1946), another at the end of the outbreak (C168 on August 6, 1946), and the last from a single sporadic case approximately 3 months later (C181 on December 3, 1946). This agent when inoculated into guinea pigs and in animals of all sizes regularly produced febrile reactions which began 3 to 5 days after inoculation and lasted 2 to 4 days

* Presented in abstract form before the 28th Conference of Research Workers in Animal Diseases in North America, Chicago, Illinois, December 2, 1947.

(Text-fig. 1). These effects were observed consistently for 3 strains carried through 5 serial passages, for another through 25 passages, and for still another through more than 50 continuous transfers. No additional signs of infection were noted, with the exception of a slight loss of weight during the febrile period which never exceeded 10 per cent of the total body weight. Five guinea pigs recovered from infection with each strain were given second inoculations with the homologous strain, and all of these animals proved immune. Similar groups of 5 guinea pigs each were then inoculated with different strains in complete reciprocal immunity tests. Each strain immunized against all of the others.

Guinea pigs killed during the febrile period showed on autopsy scattered petechial hemorrhages in the lungs and minute white spots in the liver. His-



TEXT-FIG. 1.

topathological examination of the lungs revealed alveoli filled with extravasated blood (Fig. 1). Liver lesions represented areas of liver cell necrosis midzonally located (Fig. 2); while sections of adrenals, spleen, heart, and kidneys showed no evidence of infection. Other guinea pigs killed after recovery showed essentially negative autopsy findings, with pigmented areas in the lungs as the only evidence of previous lesions.

Two uninoculated guinea pigs were placed in the same cage with 2 others each of which had been given intraperitoneally 1 cc. of defibrinated blood from infected guinea pigs. The two inoculated animals were allowed to develop illness and to recover before their removal from the cage. The contact animals after an observation period of 14 to 21 days failed to show signs of illness. They were then given the usual inoculation in a test for immunity. None were immune. This experiment was repeated 4 times with consistent results, which indicated that in guinea pigs the disease did not spread by contact.

Results obtained thus far indicated that an infectious agent had been secured in guinea pigs. Inoculation with this agent regularly caused definite signs of illness and lesions in the animals, regardless of size, and the disease did not spread by cage contact. Also, the infection resulted in complete immunity. A test for the agent therefore consisted of an initial intraperitoneal inoculation of guinea pigs and an observation period of 14 days, followed by a challenge inoculation with an established strain. This test was used in all of the work that follows.

Eggs.—In attempts to transfer the agent to 8, 9, or 10 day embryonated eggs inoculations were made on each chorioallantoic membrane with 0.1 cc. of blood from infected guinea pigs. Serial transfer was conducted by inoculating as for original passage with either a 10 per cent suspension of chorioallantoic membrane in saline or chorioallantoic fluid. In all, 3 strains were tested in this manner. After 10 serial passages tests with each strain showed that the agent was present in 10 per cent suspensions of chorioallantoic membrane in saline, in 10 per cent embryo suspension in saline, and in chorioallantoic fluid. Serial transfers were continued with one strain through 50 passages and subsequently chorioallantoic fluid was inoculated into guinea pigs and calves. Typical signs and lesions were produced. As an additional test guinea pigs recovered from infection produced with this egg passage material were inoculated with the agent that had been maintained for more than 50 continuous transfers in guinea pigs. These animals proved immune. Inoculated eggs showed no definite signs of infection in early passages (1st to 10th) but thereafter most embryos died 7 days after inoculation, although they appeared to develop normally for 5 days.

Mice.—Each of a group of 5 mice was inoculated intraperitoneally with 1 cc. of infected chorioallantoic fluid that had been transferred serially for 8 passages. Five days after inoculation the mice were autopsied. A 10 per cent suspension of pooled spleens was prepared and inoculated into another group as described above. The presence of the agent could be demonstrated after 5 serial passages in mice although all mice remained apparently normal and no lesions were found on autopsy.

Rabbits.—Each of 2 rabbits was inoculated with 1 cc. of infected chorioallantoic fluid that had been serially transferred for 8 passages. Five days after inoculation 5 cc. of blood was removed by heart puncture from each rabbit, pooled, and 2 other rabbits were inoculated with it as described above. After 5 serial passages tests showed the agent present in the blood of the inoculated rabbits. Temperature reactions similar to those obtained in guinea pigs were shown by all inoculated rabbits in each passage. Autopsies made during the febrile period on one rabbit from each series disclosed no gross lesions.

Miscellaneous Animals.—Inoculations were made subcutaneously with 1 cc. chorioallantoic fluid from infected eggs into each of 3 puppies (2, 4, and 8 weeks

old) and into 1 pig weighing 30 pounds. None of these animals showed increased temperature or other signs of illness. Each of a group of 6 hamsters was inoculated intraperitoneally with 1 cc. of defibrinated blood from infected guinea pigs; the strain used had been serially transferred through 89 passages. Of this group 3 died 9 to 12 days after inoculation. Autopsies showed hemorrhages in the lungs. The remaining hamsters showed pale shrunken kidneys when killed 25 days after inoculation. Similar results were obtained from another group of hamsters inoculated in the same manner with material that had been serially transferred through 12 passages. Tests for the infective agent were not made on any of these animals.

Nature of the Etiological Agent

Properties.—The greatest concentration of the agent was found in the chorioallantoic fluid from infected eggs 5 to 7 days after inoculation and this never exceeded 10,000 infective doses per cc. for guinea pigs. Using material of this sort, tests showed that the agent did not survive lyophilization and that freezing with dry ice caused at least a thousandfold loss in activity, while continued storage for 6 months under dry ice refrigeration resulted in complete loss of activity. Centrifugation at a speed of 24,000 R.P.M. did not remove the infective agent from the supernatant fraction of the chorioallantoic fluid from infected eggs. In 2 separate tests of this latter, the agent was found to pass through Berkefeld N filters.

All attempts failed to demonstrate a cultivable agent in defibrinated blood from infected guinea pigs, or in chorioallantoic fluid from infected eggs, by inoculation of blood agar slant and sealed cooked meat medium incubated at 37°C. Additional cultures made of chorioallantoic fluid from infected eggs in Fletcher's medium showed no growth when incubated at room temperature (approximately 22°C.). Films of defibrinated blood from infected guinea pigs and chorioallantoic fluid from infected eggs stained by methylene blue, Gram's method, Giemsa's method or Macchiavello's method showed no visible forms.

Demonstration of Spirochetes.—Preparations of blood and kidneys from infected guinea pigs infective for other animals showed no organisms upon dark-field examination ($\times 160$). We are indebted to Dr. J. B. Nelson for staining the preparations of chorioallantoic fluid from infected eggs by Morosow's method, thus permitting the first demonstration of spirochetes. Later dark-field examinations of chorioallantoic fluid from some infected eggs showed in each field 3 to 5 spiral-shaped organisms that rotated rapidly on the long axis. In general, organisms were more readily found in eggs 5 days after inoculation and before death of embryos than 7 days after inoculation or following death of the embryos. A few forms were found also in blood from calves inoculated with chorioallantoic fluid from infected eggs. Examination of the urine from these calves showed no organisms although it was capable of infecting guinea pigs.

Sections of kidneys from 5 calves inoculated with either chorioallantoic fluid from infected eggs or defibrinated blood from infected guinea pigs were stained by Levaditi's method but showed no organisms. Films of blood and kidneys from infected guinea pigs stained by Morosow's method showed no organisms. Preparation of chorioallantoic fluid from infected eggs or cultures similarly treated showed a few spiral-shaped forms (Fig. 3).

Chorioallantoic fluid from infected eggs, blood from infected guinea pigs, blood from infected rabbits, blood from experimentally infected calves, and urine from experimentally infected calves after centrifugation at 10,000 R.P.M. for an hour were inoculated into tubes of semisolid meat infusion agar that contained either 10 per cent rabbit, calf, or horse serum. These cultures then were placed at 37°C., 30°C., or room temperature (approximately 22°C.). Darkfield examinations made 3 days later showed a few spiral-shaped organisms in the tubes incubated at 37°C. After 2 weeks, examination of tubes placed at 37°C. revealed a diffuse growth in the top portion of the medium. Growth occurred also at 30°C. although it was more slow and required a month for similar development. No visible growth occurred at room temperature.

The spiral-shaped organisms growing in culture were examined with the electron microscope. Measurements showed a diameter of 90 millimicrons and a length, commonly of 4 microns (Fig. 4), but varying from 4 to 16 microns according to the number and depth of spirals. No internal structure was revealed.

Cultures of suspensions of organisms heated at 50°C. for 10 minutes showed no growth while those heated at 45°C. for 10 minutes grew. In 2 attempts, growth was obtained from Berkefeld N filtrates of a suspension of organisms. A suspension of previously motile organisms showed no activity after freezing with dry ice. Five guinea pigs which had been inoculated with 1 cc. of cultured organisms transferred 5 times in media were reinfected 14 days later with 1 cc. of chorioallantoic fluid from infected eggs. All were immune. Similarly 5 guinea pigs that had recovered from an infection produced with the agent maintained in eggs were immune when tested with cultured organisms.

Since the spirochete recovered in culture possesses physical and immunological properties similar to the infective agent, it appears legitimate to assume that it came from the sick cows originally studied.

The Experimental Disease in Cattle

Production.—Many of the cattle used came from an experimental herd maintained by this department. Repeated observations on these animals showed no evidence of this or any other infection. Some animals were purchased locally and observed for at least a week before use. In all, 5 lactating cows and 18 calves of Brown Swiss, Guernsey, Jersey, and mixed breeds were used.

Three of the cows were in their first milking period while the other 2 had had multiple lactations. The calves ranged in age from 8 days to 3 months. All

experiments were conducted upon single animals kept in strict isolation unless contact experiments were planned. Daily temperatures were taken for a week before inoculation in order to obtain an indication of each animal's normal range.

Various methods were used in attempts to produce the experimental disease: (c) subcutaneous injection of 1 cc. of defibrinated blood from infected guinea pigs (1 to 40 passages), (d) subcutaneous injection of 1 cc. of chorioallantoic fluid from infected eggs (5 to 50 passages), (e) subcutaneous injection of 1 cc.

TABLE I
Production of Disease in Cows and Calves

Method of inoculation	Inoculum	No. of animals inoculated*	Results	
			No. of animals showing infection†	No. of animals showing signs of illness
Subcutaneous	Blood from infected guinea pigs	2 cows 3 calves	2 3	5
	Chorioallantoic fluid from infected eggs	1 cow 4 calves	1 4	5
	Culture of spirochete	5 calves	5	5
	Chorioallantoic fluid from infected eggs	5 calves	5	5
Intranasal	Chorioallantoic fluid from infected eggs	5 calves	5	5
Feed	Chorioallantoic fluid from infected eggs	2 calves	0	0
Contact		3 cows 2 calves	2 1	0

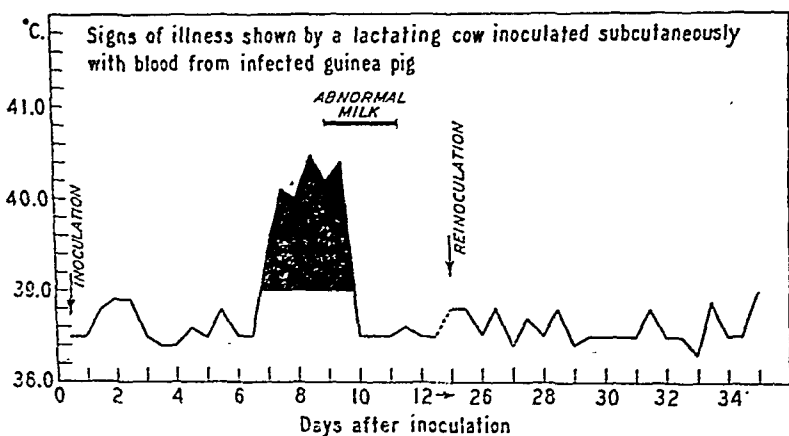
* Four animals counted twice.

† Infection based on signs of illness, recovery of infective agent, and/or immunity to subsequent injection subcutaneously.

of semisolid culture medium that contained spirochetes serially transferred for 1, 2, 5, 6, or 7 passages. (d) intranasal injection of 10 cc. of chorioallantoic fluid from the 6th to 16th serial passage in eggs, and (e) feeding of 25 cc. of chorioallantoic fluid from the 10th to 40th serial passage in eggs mixed with 1 liter of milk. Infectivity of all inocula was checked by inoculating 1 cc. intraperitoneally into a guinea pig. Contact experiments were made by placing normal animals in pen contact with infected ones. All animals were checked daily for increased temperature or other signs of illness. Animals which remained apparently normal were given a second inoculation subcutaneously with material from either infected guinea pigs or eggs. These results are summarized in Table I.

As can be seen in Table I, all 15 animals inoculated subcutaneously showed signs of illness. Three of 5 calves showed signs of illness following intranasal inoculation. The other 2 showed no signs of illness but were immune to a second inoculation given subcutaneously. Likewise 3 of 5 contact animals showed no signs of illness following exposure but subsequently were immune to subcutaneous inoculation, indicating that in these instances inapparent infection must have occurred. The infection was not transmitted in 2 contact and in 2 feeding experiments.

Features of the Illness.—Two lactating cows inoculated subcutaneously showed fever after an incubation period of 7 to 9 days. The findings in one of these animals are given in Text-fig. 2. Although these cows under experimental



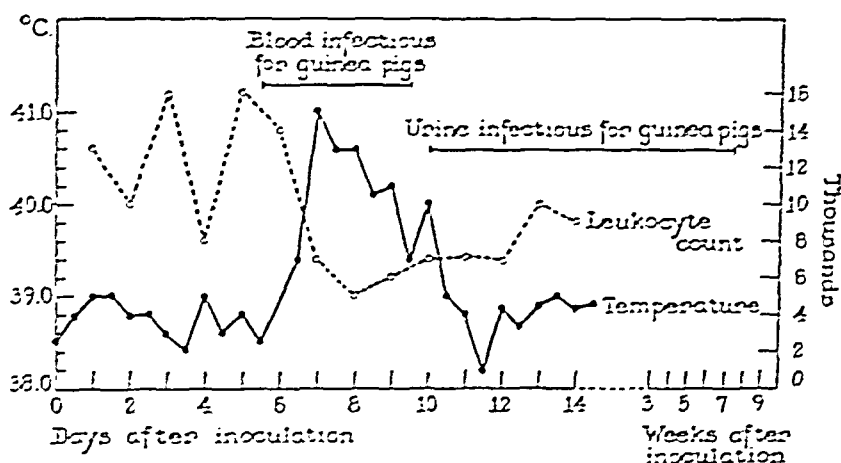
TEXT-FIG. 2.

conditions had low milk yields, the amount of milk was decreased further during the febrile period to approximately one-half that obtained before inoculation. In addition the milk became thickened, yellow in color, and contained flakes that represented collections of leucocytes. Blood as observed in natural cases could not be seen in any of the milk samples but in other respects the samples resembled those obtained from naturally infected cows. In contact experiments 2 cows placed with infected suckling calves showed no signs of illness or alterations in the milk although one animal later proved to be immune. Another cow placed in contact with naturally infected animals also showed no signs of illness or alterations in the milk but developed immunity.

Among 18 calves infected by subcutaneous or intranasal inoculation or by contact, 3 showed no signs of illness, 12 developed fever only, while 3 showed a febrile reaction and later died. Except for those with inapparent infections, a marked increase in temperature occurred 2 to 7 days after inoculation and lasted from 1 to 3 days. During the febrile period a slight anorexia was noted. All animals appeared normal following termination of fever except those that

later died. These animals became rapidly and progressively worse, showed a subnormal temperature, and died within 1 to 2 days. A hemoglobinuria signaled the approach of death.

For additional studies, specimens of blood from 5 animals were examined for total red cells and leucocytes before, during, and after illness. At the same time urine was collected and tests were made for albumin and microscopic examination of centrifuged sediment. These results are shown graphically in Text-fig. 3 for a calf and are typical of those studied. All calves showed a mild leucopenia during the febrile period. On the day that the temperature became normal, the urine showed albumin and large numbers of leucocytes which were found in the centrifuged sediment.



TEXT-FIG. 3. Features of illness shown by calf C188 inoculated intranasally with chorio-allantoic fluid from infected eggs.

Pathology.—At the end of fever 7 calves were examined: 3 that died from the infection and 4 that were killed. All organs seemed normal except the kidneys which appeared congested. Perirenal edema was noted in 2 calves. In addition the kidneys of 4 calves showed small white spots diffusely scattered over the surface. These spots, frequently irregular in outline, were approximately 1 mm. in diameter and appeared continuous with the normal surface. Section showed these spots extending either as wedges or as plugs into the cortical layer. The medullary portion appeared normal. The bladder and its contents were not unusual except in the animals that died. Collections of reddish colored urine were noted. One lactating cow and 6 calves that showed signs of illness were held for 14 days, given a second inoculation, and then killed after a further observation period of 14 days. Autopsies showed lesions in the kidneys similar to those seen in the early stages of the disease. Two calves that showed signs of illness were held 60 days after inoculation, then killed and

examined. White spots were found in the kidneys of one animal while the other showed no lesions.

A group of one lactating cow and 3 calves that showed no signs of illness was killed 14 days after a second inoculation. Autopsies showed no lesions.

A similarity of lesions in the kidneys among cows and calves in all stages of infection was found on histological examination. Widespread but focal involvement of the cortex and corticomedullary junction (Fig. 5) was noted and consisted of irregularly shaped patches of infiltrating cells predominantly mononuclear but mixed with varying numbers of eosinophilic polymorphonuclears (Fig. 6). Most tubules were destroyed in these patches. Tubules in the affected areas and at some distance away frequently contained masses of cells identical with those forming the infiltrates. Near the patches of cellular infiltrates mononuclear cells were seen between tubules in areas normally occupied by peritubular capillaries. Glomeruli in general appeared normal with patent vascular coils, but there was some evidence of capillary damage indicated by deeply staining eosinophilic nuclei and leakage of protein into capsular spaces. Tubules throughout the cortex were generally well preserved except in areas of cellular infiltration. Tubular lumina, however, contained in many cases protein precipitates. Here and there proximal tubules showed smaller cells without nuclei and with a finely vacuolated cytoplasm. The distal and intermediate tubules seemed less affected. There were no significant changes in collecting tubules.

Presence and Persistence of Spirochetes in Inoculated Animals.—Three cows were inoculated subcutaneously, 2 with blood from infected guinea pigs and the other with chorioallantoic fluid from infected eggs. Tests made at the time of inoculation and during the subsequent febrile period showed the presence of the agent in both blood and milk during the febrile period in one of the cows that had been inoculated with blood from infected guinea pigs. The agent was not recovered in either blood or milk from the other 2 cows. In similar tests made on blood, milk, and urine from a naturally infected cow during the acute phase of illness, the agent was recovered from milk samples only.

Tests made on all calves during the febrile period showed the agent in blood. From 2 calves, one inoculated subcutaneously with 1 cc. of defibrinated blood from an infected guinea pig and the other inoculated intranasally with 10 cc. allantoic fluid from infected eggs, tests for the agent were made daily on blood and urine from the time of inoculation through the febrile period and at 2 week intervals thereafter. These results, presented graphically in Text-fig. 3 for one calf, showed the agent present in the blood 1 day before the onset of fever and during the febrile period. On the day the temperature became normal, the blood no longer contained the infective agent which, however, persisted for 53 days in the urine. The other calf responded in a similar fashion. Additional studies on 3 calves during the incubation period, the febrile period, and a few

tana, Johnson (12) in Australia, and Mathews (13) in Texas, the diagnosis was made after autopsy by staining tissue sections of diseased cattle to identify the leptospira. Certain clinical types of these leptospiral infections appeared identical with the natural disease observed in this outbreak but differed in most cases. Most significant was the fact that no abortion or icterus occurred and hemoglobinuria was rare although these features as well as the presence of leptospira in the kidney were reported by other workers. Since in our preliminary studies we were unable to demonstrate leptospira microscopically in kidney sections or culturally by the usual means of cultivation in Fletcher's medium at room temperature and since there were additional differences as well, further search was made for the etiological agent.

It was shown that the agent is a spirochete readily transmissible to guinea pigs, rabbits, embryonated eggs, mice, lactating cows, and young calves. Experimentally produced infection in the natural host resulted in a variation of obvious findings that ranged from a few cases of inapparent infection with normal urine through the usual condition of fever with albuminuria to occasional hemoglobinuria and death. These manifestations may explain the observations in the original outbreak of instances of hemoglobinuria and also the occasional animal in contact with natural cases that developed immunity without evidence of disease. The spirochete is not confined to the mammary gland but causes a generalized infection with subsequent localization in the kidney. In this organ the lesions and the infectious agent persisted for nearly 2 months, long after the latter had disappeared from the blood and after immunity had become established.

The following facts have a bearing on the spread of this disease. There is strong reason to believe that some animals undergo a form of natural nasal inoculation. Urine that contains the spirochete may be excreted from a standing animal onto a concrete barn floor, thereby causing a spray of droplets some of which in turn could be inhaled by nearby animals. This mode of infection might explain how a single animal could initiate the disease in a susceptible herd. This could have occurred in the outbreak herein reported, since the usual method of herd replacement was followed by constantly bringing in large numbers of animals from various regions of the United States.

The leptospiras from cattle found in Russia, Palestine, and Australia have been reported to cause disease in man. This has not been shown for the spirochete studied in the present paper but, since this organism is present in milk, infection might occur through ingestion, although experimental calves did not become infected by this route.

The agent was present in the blood during early stages and infection was produced by inoculation subcutaneously. Insect vectors therefore must be considered as a possible epidemiological factor and a search should be made for the reservoir hosts.

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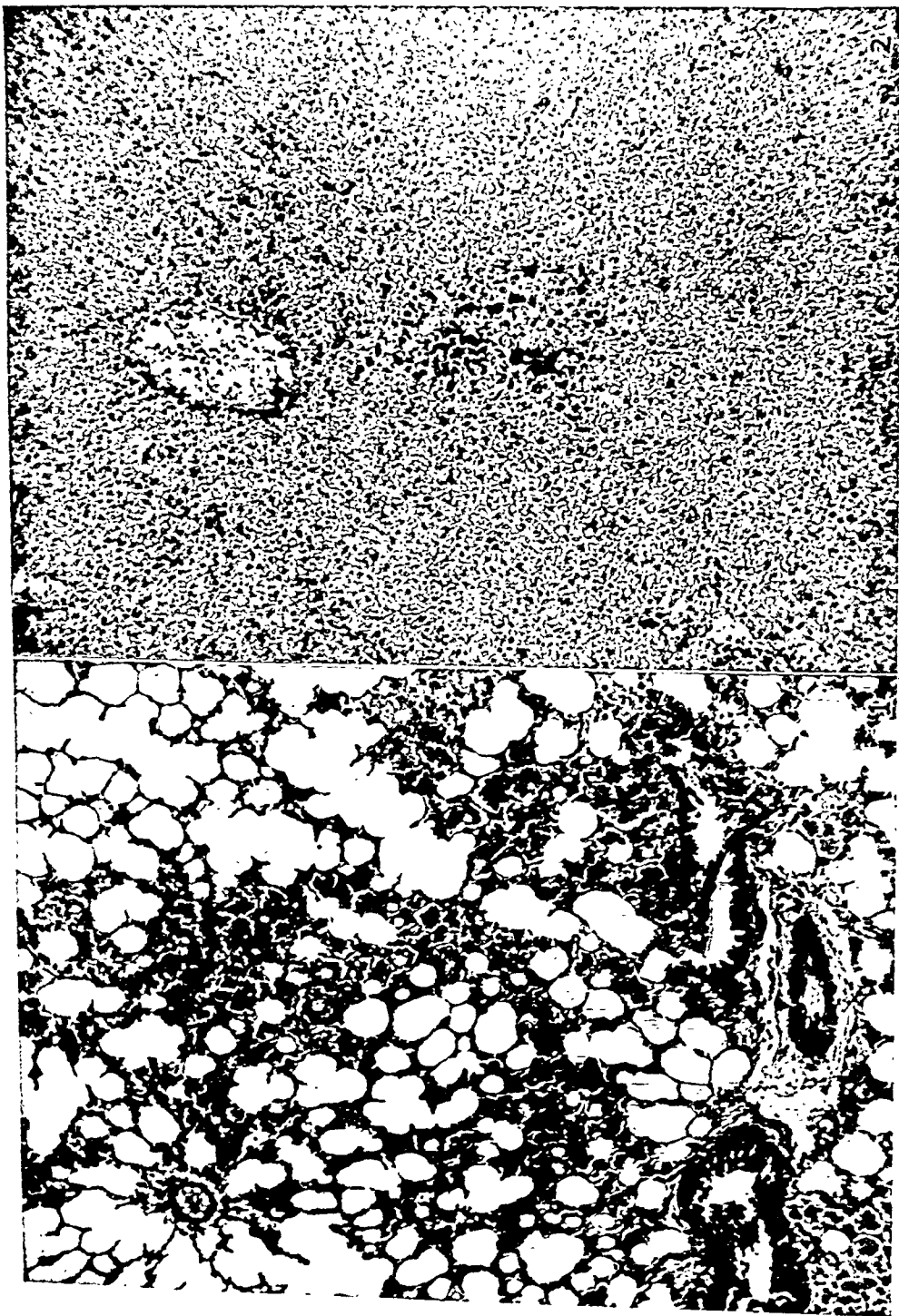
EXPLANATION OF PLATES

PLATE 10

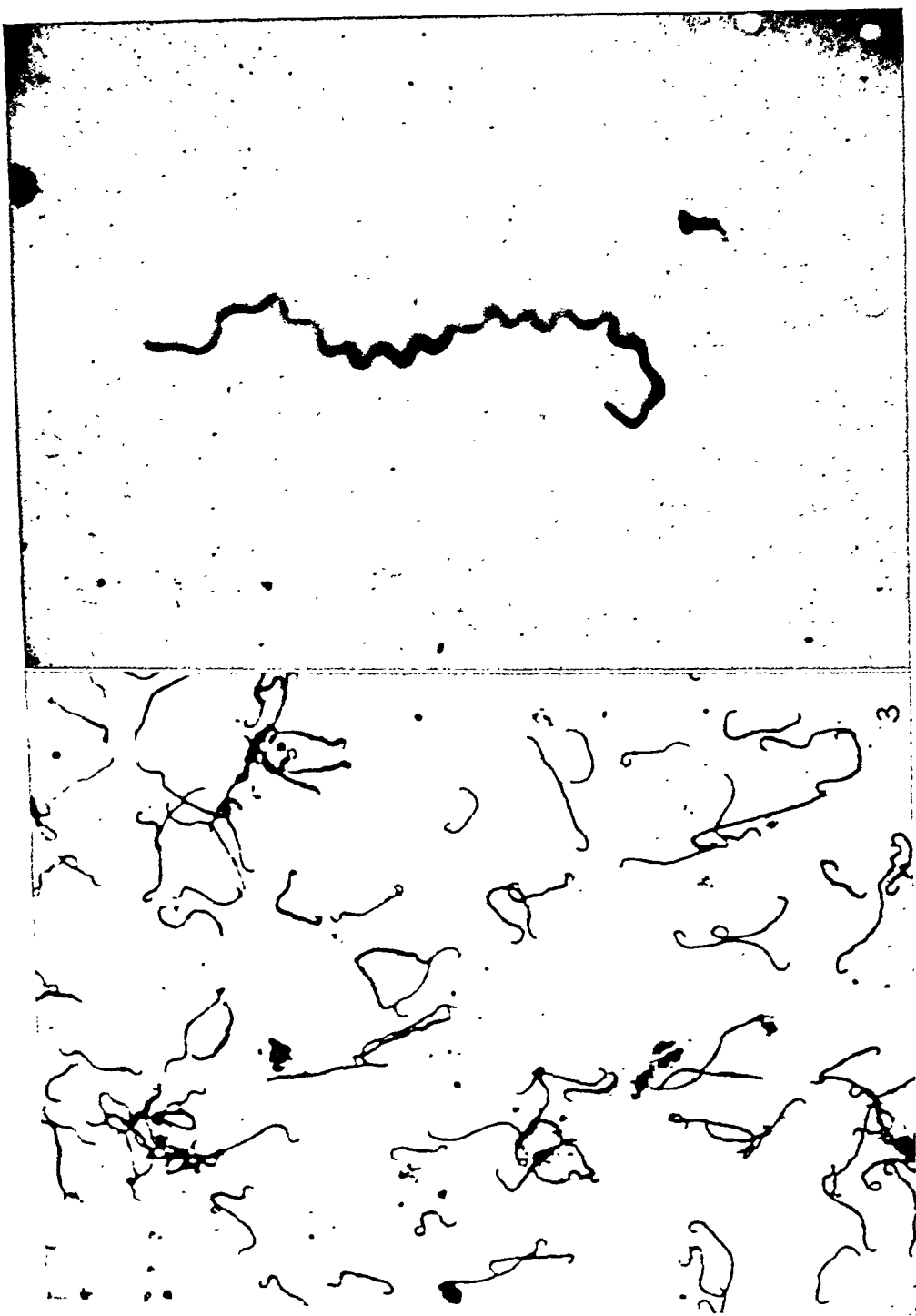
Photographs by Mr. J. A. Carlile.

FIG. 1. Lung from infected guinea pig showing hemorrhage into alveoli. Stained with polychrome methylene blue. $\times 112$.

FIG. 2. Liver from infected guinea pigs. Note area of necrosis. Stained with polychrome methylene blue. $\times 112$.



(Baker and Little: Leptospirosis in cattle)



(Baker and Little: Leptospirosis in cattle)

PLATE 12

FIG. 5. Kidney from infected calf killed 28 days after inoculation. Stained with polychrome methylene blue. $\times 112$.

FIG. 6. Kidney from infected calf. Note cellular infiltration and tubular damage. Glomerulus appears normal. Stained with polychrome methylene blue. $\times 688$.



(Baker and Little: Leptospirosis in cattle)

EPIDEMIC DIARRHEAL DISEASE OF SUCKLING MICE

III. THE EFFECT OF STRAIN, LITTER, AND SEASON UPON THE INCIDENCE OF THE DISEASE*

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An epidemic diarrheal disease of 2 to 3 weeks old suckling mice in the breeding colony of this laboratory has been described in previous papers (1, 2). No recognized bacterial pathogen was isolated from these animals and inflammatory changes in the large and small bowel were minimal or absent. The finding of intranuclear inclusion bodies in the epithelial cells of the villi of the small intestine (2) strengthened the supposition that the disease might be fundamentally one of viral origin. Before this theory could be subjected to adequate experimental trial an unfortunate accident in the animal house wiped out practically the entire colony.

During the course of our work it was brought to our attention that diarrheal disease of suckling mice was endemic in the colonies of several other breeders. It was determined to introduce one or more of these strains of mice into our laboratory in order to study the occurrence of the diarrheal syndrome in these animals with an eye to determining whether the etiology of the disease was single or multiple. Furthermore it was of primary importance to determine the relative resistance or susceptibility of the various strains; for if a highly susceptible strain were discovered it would greatly facilitate the carrying out and evaluation of transmission experiments. Conversely the finding of a relatively resistant strain would permit us to supply the number of mice required by the other activities of the laboratory with a minimum of loss.

EXPERIMENTAL

The only survivors of our original (Harvard) stock that remained after the accident in the Animal House were nine females and one male which had been transferred prior to the event to an isolated room in the Medical School. Here they had been kept in close contact with a litter of strain C white mice obtained from the National Institute of Health, Bethesda, Maryland, through the kindness of Dr. H. B. Andervont. Diarrheal disease had been endemic among the suckling mice of this colony for some time. When it was realized that further work under conditions identical with those that had prevailed in 1944-45 was impossible because the few survivors of the original stock had already been exposed to the possibility of superinfection from an extraneous source, it was decided to introduce additional strains of mice without further delay in order to broaden the scope of the experiment. The four strains finally selected were:

* Aided by a grant from The National Foundation for Infantile Paralysis.

during the 2nd and 3rd weeks of life were obviously due to the effects of the diarrheal disease, the percentage of mice of any given litter that were weaned formed a fair index of the severity of the process. Mice dying during the 1st

TABLE I
Per Cent of Mice Weaned, by Strain

Strain	Born	Weaned	Per cent
Schwentker.....	924	546	59.1 \pm 1.62
Harvard.....	821	589	71.7 \pm 1.57
C.....	845	527	62.4 \pm 1.67
CFW.....	987	501	50.8 \pm 1.59
Total.....	3,577	2,163	60.5 \pm 0.82

TABLE II
Per Cent of Mice Weaned, by Litter

Litters	Born	Weaned	Per cent
1st.....	603	221	36.7 \pm 1.97
2nd and 3rd.....	1,599	956	59.8 \pm 1.22
4th and 5th.....	1,375	986	71.7 \pm 1.41
Total.....	3,577	2,163	60.5 \pm 0.82

TABLE III
Per Cent of Mice Weaned, by Month

Period	Born	Weaned	Per cent
Aug.-Sept., 1946.....	144	125	87.7 \pm 2.74
Oct.-Nov., 1946.....	654	240	37.1 \pm 1.89
Dec.-Jan., 1946-47.....	997	606	60.8 \pm 1.55
Feb.-Mar., 1947.....	868	469	54.0 \pm 1.69
Apr.-May, 1947.....	604	454	75.1 \pm 1.76
June-July, 1947.....	252	222	88.1 \pm 2.04
Aug.-Sept., 1947.....	58	47	81.0 \pm 5.15
Total.....	3,577	2,163	60.5 \pm 0.82

week of life were excluded from the calculations. To the number of mice dying between the 7th day and the 22nd day of life (the time at which the young mice were weaned) were added those relatively few mice that had to be sacrificed at the time of weaning because of the presence of severe obstipation, the commonest complication of the disease. This figure represented the total number

of deaths attributed to the disease. To it was added the number of mice successfully weaned from each litter in order to obtain the total number of mice exposed to the risk of death due to the disease.

Since the purpose of the experiment was to determine the effect of strain of mice, of litter (first as opposed to later ones), and of season upon the mortality of the weanling mice, the data were first analyzed from these points of view.

TABLE VI
Per Cent of Mice Weaned, by Litter
(Strain and date constant)

Month	1st Litter mice weaned	2nd and 3rd litter mice weaned	4th and 5th litter mice weaned
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
<i>Schwentker</i>			
Oct.-Nov., 1946.....	8.8	56.5	
Dec., 1946-Jan., 1947.....		81.8	90.0
<i>Harvard</i>			
Oct.-Nov., 1946.....	45.7	42.5	
Dec., 1946-Jan., 1947.....		68.5	97.4
Feb.-Mar., 1947.....		60.4	83.5
<i>C</i>			
Oct.-Nov., 1946.....	38.9	57.1	
Dec., 1946-Jan., 1947.....	41.4	52.8	63.5
Feb.-Mar., 1947.....		78.5	50.0
Apr.-May, 1947.....		46.2	81.7
<i>CFW</i>			
Oct.-Nov., 1946.....	0.0	21.0	
Dec., 1946-Jan., 1947.....	3.1	23.5	45.9
Feb.-Mar., 1947.....	0.0	53.1	56.3
Apr.-May, 1947.....		65.1	74.7
June-July, 1947.....		92.0	84.4

In Table I, the percentages of mice weaned are given by strain. The Harvard strain appears to be the most resistant and the CFW mice the least so; the individual differences noted between these four groups are statistically significant except in the case of the Schwentker and C strains.

When grouped according to litter as in Table II, significant differences appear between first litters as opposed to second and third, and to fourth and fifth litters. Approximately 1 of 3 suckling first litter mice survived to be weaned, slightly better than 1 of 2 second and third litter mice, and nearly 3 of 4 fourth and fifth litter mice. These differences are in agreement with our previous experience.

litters the differences between strains are less consistent and frequently are not statistically significant.

When strain of mice and date of birth are comparable the influence of litter upon percentage of mice weaned (Table VI) is less marked but still reasonably consistent, and many of the individual differences are statistically significant. For all strains at nearly all times of the year the mortality in first litter mice was higher than in second and third litter ones, which in turn presented a higher mortality than did fourth and fifth litter mice.

When the mice are grouped according to date of birth with strain and litter comparable (Table VII) there is in the case of the CFW strain a fairly consistent pattern which bears a close resemblance to that of Table III. A high percentage of the mice born in the summer of 1946 survived to be weaned. In the autumn of 1946 the percentage dropped precipitously. The periods of Feb. to Mar., 1947, and Apr. to May, 1947, showed a gradual rise in the percentage of mice weaned. Again a low mortality was observed during the summer months of 1947. Although but few of the individual differences are statistically significant the general trend is quite consistent. In the case of the other strains the evidence is less satisfactory. The strain C mice present a picture somewhat similar to that shown by the CFW strain but the trend is less consistent and the individual differences smaller. The data furnished by the Harvard and Schwentker strains are too fragmentary to permit the drawing of conclusions.

DISCUSSION

Our investigations have clearly confirmed the report that suckling mice belonging to the colony of the C3H strain were highly susceptible to diarrheal disease. The stock was freed of detectable infection by the delivery of the young by Caesarian section at term, and the subsequent nursing of these animals by presumably disease-free females belonging to another strain under conditions of rigid isolation. This colony is increasing slowly in size and has remained free of disease for nearly a year.

Among the four strains of mice that were observed during the course of a year significant differences were limited to the CFW strain. A lower percentage of these mice were weaned fairly consistently in comparison to the others. The three more resistant strains failed to show consistent or significant differences in the percentage of mice weaned.

The effect of litter was definite and consistent. For all four strains followed throughout the course of a year first litters fared worse than second and third ones while fourth and fifth litters showed the highest percentage of mice weaned. A possible explanation for this finding is that the breeding females may develop some degree of active immunity against the disease as a result of sustained contact with it, and that this immunity may be passed on in some degree to the young either *in utero* across the placental barrier or by the milk or colostrum.

EPIDEMIC DIARRHEAL DISEASE OF SUCKLING MICE

IV. CYTOPLASMIC INCLUSION BODIES IN INTESTINAL EPITHELIUM IN RELATION TO THE DISEASE*†

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In a previous report (1), Pappenheimer and Enders described the occurrence of intranuclear inclusions in a large proportion of suckling mice with diarrhea (Fig. 1). The original stock in which the spontaneous disease had been prevalent for several years was virtually destroyed during the summer of 1946. Since then, new stock from several sources has developed spontaneous diarrhea, indistinguishable, as far as obvious signs are concerned, from the disease as it appeared in the original stock. The epidemiology of the disease as now prevalent is described in the preceding paper (2). In no instance have we succeeded in demonstrating intranuclear inclusions of the type previously described. However, many of the mice have had *cytoplasmic* inclusions in the epithelial cells of the small intestine.

In this study, evidence is presented bearing on the possible relation of these cytoplasmic inclusion bodies to the diarrheal disease as it has occurred in our colony subsequent to the introduction of new strains.

Methods

The entire small intestine was fixed in Zenker's fluid, without acetic acid, and embedded *in toto* so that the paraffin sections passed through the gut at various levels. As routine stain for the demonstration of the inclusions, Laidlaw's acid fuchsin-phosphomolybdic acid-orange G method has proven most satisfactory, although the bodies are also easily found in hematoxylin-eosin and in Giemsa-stained preparations.

Appearance of the Inclusion Bodies (Fig. 2).

The bodies are spherical, varying in size from 1 to 4 micra in diameter, sharply outlined, and sometimes, but not regularly, surrounded by a narrow clear halo. With the Laidlaw stain, they are intensely fuchsinophilic. In the larger forms, the center may appear rarefied. The position in the cell varies; the bodies may be found above, below, or to one side of the nucleus. There is usually only a single inclusion body to a cell, but occasionally, there may be

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required for their detection, so that failure to find them in some cases does not necessarily mean that they were not present.

With the Laidlaw stain, the granules of Paneth's cells stain intensely with acid fuchsin, and are very resistant to decolorization with alcoholic orange G. Their location at the base of the villi, and a slight difference in color, the Paneth granules having a slight purplish cast as compared with the vermillion red color of the cytoplasmic inclusions, enable one to distinguish them without difficulty.

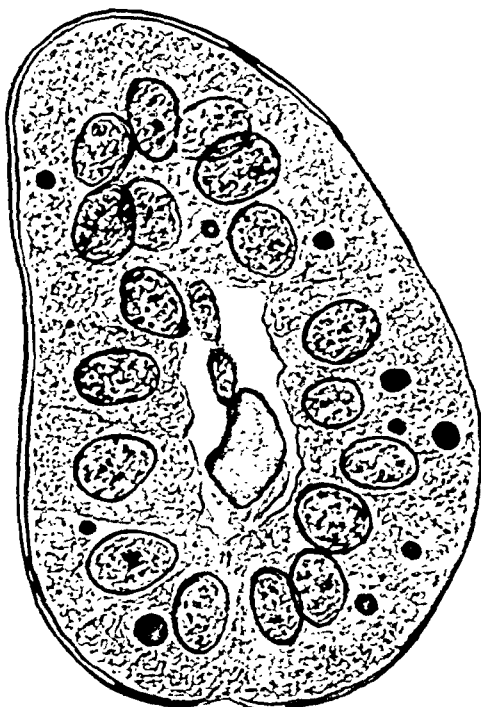


FIG. 2. Cytoplasmic inclusions in epithelial cells of small intestine. Laidlaw stain. $\times 1000$.

More confusing is the frequent occurrence, especially in healthy mice a week or less old, of a somewhat different type of cytoplasmic inclusion. This was described and pictured in a previous report (1). It stains with Laidlaw, Giemsa, and eosin much as does the inclusion body described above, but with the Laidlaw stain, it holds the fuchsin much less tenaciously upon decolorization with orange G. The average size is much larger; the shape tends to be oval rather than spherical; it is surrounded by a wide clear space, and it is always located above the nucleus towards the intestinal lumen. When present, practically every cell of the villus contains such a body, and there is never more than one to a cell.

Although there is nothing to give a clue as to the significance of the type of body just described, it can usually be clearly differentiated from the cytoplasmic inclusions with which we are particularly concerned in this study. The dif-

In a third group, consisting of 42 mice, the crude extract was rendered bacteria-free by treating it with penicillin and streptomycin. The mice developed mild diarrhea; many had recovered when they were sacrificed at 21 days. Cytoplasmic inclusions were found in 8 of the 42 mice.

In summary, the incidence of inclusions in 64 mice with diarrhea was 11 or 17 per cent, whereas in the 21 mice which showed no signs of the disease, it was 47 per cent. There appeared to be no correlation whatever between the occurrence of diarrhea and the presence of cytoplasmic inclusions.

It should be said that with one or two exceptions, the inclusion bodies were very scarce, and often found only after long study of the sections. In a number of mice, they were seen only in castoff cells lying free in the lumen. It is probable that examination at an earlier stage before the affected cells had been exfoliated, would have yielded quite different results. Nevertheless, the presence of inclusions in a fairly large percentage of mice which had been fed with heated suspension and had failed to develop the disease, threw doubt upon their significance as a specific feature of the disease. There remained however, the theoretic possibility that the mice had acquired an inapparent infection while they were under observation. Since one of the four litters given heated extract did actually develop diarrhea in the course of the experiment, this possibility could not be excluded.

Experiment 2.—Four litters, in all comprising 26 mice, were fed crude suspension of diarrheal feces, at the age of 7 and 8 days, and were sacrificed 5 days later. At this time, two of the four litters showed the usual signs of diarrhea, two litters remained apparently well. Cytoplasmic inclusions were found in 22 of the 26 mice (85 per cent); they were present in 10 mice which did not have signs of diarrhea, although they had been given potentially infective material. This suggests that these may have had the disease in inapparent form.

As controls, 36 mice received a suspension of feces from normal stock which has been free of the disease, and has been maintained in a separate room under special precautions. None developed diarrhea, and in none were cytoplasmic inclusions found.

The experiment indicates that the feces of normal mice do not contain the factor responsible for the cytoplasmic inclusions.

Experiment 3.—Nine mice from three different litters were fed crude extract, and 9 mice were given boiled extract. One mouse from each litter was examined after 2, 3, and 6 days. Of those given crude extract, 5 developed diarrhea; those receiving boiled extract remained well.

In the former group, 6 of the 9 showed cytoplasmic inclusions; they were found also in 2 of the 9 mice which had received the boiled extract, though in small numbers and only after prolonged search.

Experiment 4.—To eliminate the possibility that some of the mice might have become infected while under observation, the experiment was repeated. The mice receiving the heated extract were kept isolated in a separate room which had been free from the disease. All the 20 mice given heated extract remained well, and in none were typical inclusions found. Seventeen mice received crude unboiled extract. Five were killed and examined before the onset

of symptoms, and showed no inclusions. The remaining 12 developed diarrhea, and in 11 of these, typical inclusions were demonstrated.

Time of Appearance of Inclusions in Relation to Diarrheal Disease (Fig. 3)

When the material from both spontaneous and experimentally produced disease is analyzed with respect to the relation between the presence of inclusions and the day of the disease, it is strikingly shown that inclusions are regularly found only in the early stages of the infection. The experimental infection is particularly informative in this respect. Whereas 32 of 33 mice

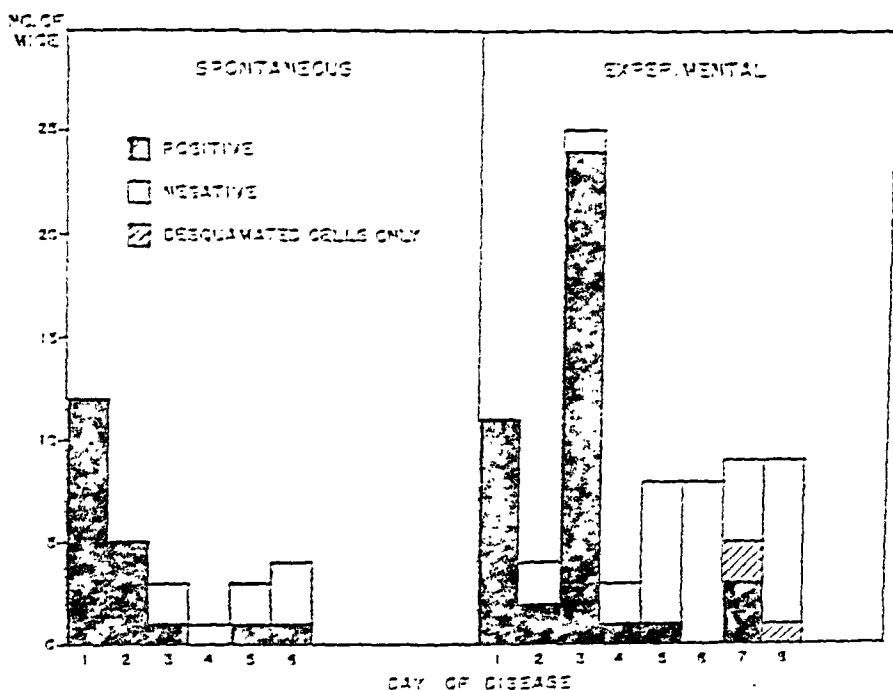


FIG. 3. Occurrence of cytoplasmic inclusion bodies by day of disease

(97 per cent) killed on the 1st, 2nd, or 3rd day of the diarrheal illness, showed inclusions, only 7 of 21 sacrificed on the 4th to the 8th day were recorded as positive; in 3 of these, the inclusions were seen only in desquamated cells lying free in the lumen. A similar difference was found in the spontaneous cases. Of 19 mice killed on the 1st and 2nd days after appearance of signs, 17 (89 per cent) had inclusions; these were present in but 3 of 11 sacrificed between the 3rd and 6th days, and only in sparse numbers.

It is thus important to search for the inclusions in the very early stages of the disease.

Gram-Positive Coccoid Bodies in the Intestinal Contents

Almost from the beginning of our studies, we have noted the presence, usually in enormous numbers, of Gram-positive coccoid bodies in the contents

of the small intestine. Although they may occur in stock mice which have shown no diarrheal symptoms, they are present with far greater frequency in the intestines of the diarrheal animals, and appear to be in some way correlated with the disease. This is brought out more clearly by our study of the experimental material. Thus, in Experiment 4, coccoid bodies were noted in 14 of 17 mice which had received crude extract and had developed diarrhea in consequence; and they were absent in 20 non-diarrheal controls given sterile heated extract. The 3 negative cases in the infected group were sacrificed on the 1st day of the disease.

The presence of these coccoid bodies can be recognized even under the low power of the microscope because, unlike the normal intestinal bacteria, they adhere in a fuzzy fringe to the epithelial surface. With the immersion lens, they are found to align themselves in a single row against the cuticular border of the cells. Unsuccessful attempts have been made to cultivate these organisms on the usual laboratory media. At this time, we wish merely to record their frequent and rather characteristic occurrence in this disease, without attempting further to assess their significance.

DISCUSSION

That there is a relation between the occurrence of cytoplasmic inclusions and the diarrheal disease of suckling mice now prevalent in the laboratory, can hardly be doubted. In the spontaneously occurring cases, inclusions were demonstrated in approximately 60 per cent; and they were invariably absent in stock of the same strain kept in a room which remained free from the infection.

In experimentally infected mice, the results are equally convincing. In 47 mice receiving crude unboiled extract of diarrheal intestine, 39 or 83 per cent were found to have typical cytoplasmic bodies. They were found in but 2 of 65 controls without diarrhea, including 36 given normal intestinal extract and 29 given boiled diarrheal extract. The 2 positive cases which occurred in the last group, we are inclined to attribute to some undetected error in labelling bottles or sections, or more probably, to the acquisition of an accidental infection during the period of observation.

It is true that the results in the first series of 87 mice were less clear cut, and that inclusions were found in a considerable number of mice that had been given boiled and presumably non-infective intestinal extract. We have no explanation for this discordant result, but the fact that one of the four litters developed diarrhea while under observation, suggests that they may have become accidentally infected in the course of the experiment. When precautions were taken to keep the animals in a non-infective environment throughout the experiment (Experiment 4), no inclusions were to be found.

The presence of inclusions in certain litters which had been given potentially infective material, but which failed to manifest signs of diarrheal disease, also

PROTECTIVE EFFECT OF HYALURONIDASE AND TYPE-SPECIFIC ANTI-M SERUM ON EXPERIMENTAL GROUP A STREPTOCOCCUS INFECTIONS IN MICE

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PLATES 13 TO 15

(Received for publication, June 17, 1948)

The pathogenicity and invasiveness of group A streptococci for man, as well as for laboratory animals, have been shown to be closely correlated with the presence of the M protein; conflicting evidence, however, has been obtained by several groups of investigators with regard to the rôle of the hyaluronic acid capsule in the manifestation of virulence of these microorganisms.

A number of studies have been carried out in an effort to ascertain the effect of these substances on the pathogenesis of streptococcal infections. Several workers have studied the protective action in mice, infected with group A streptococci, of immune serum containing antibodies directed against the M substance. Others have studied the protective effect of hydrolyzing the capsular material with an enzyme, hyaluronidase. As a result of the antibody studies the M substance has been shown to be essential for the exhibition of virulence, since anti-M antibodies provide active and passive protection of mice against infection with virulent streptococci of homologous type (1-3). On the other hand, the relation of the hyaluronic acid capsule to mouse virulence is not so well defined. While Hirst (4) was able to protect mice infected with encapsulated group C streptococci by treatment with leech extract containing hyaluronidase, he was not able to protect mice infected with group A streptococci; although the capsular materials of both these groups of streptococci have been shown by Seastone (5) to be chemically similar. Blundell (6) obtained no definite protection against group A streptococcus infections in mice with crude bovine testicular hyaluronidase but observed a greater mean survival time; and McClean (7), using enzyme from a similar source, failed to obtain protection in mice infected with either group A or group C streptococci.

However, Kass and Seastone (8), who recently succeeded in protecting mice to some extent against infection with group A streptococci by using bovine testicular hyaluronidase, ascribed the failure of earlier workers to the use of insufficient amounts of enzyme and to too long an interval between injections. Both of these factors tended to permit the streptococci to regenerate their capsules in the infected host.

In view of these conflicting reports as to the protective effect of hyaluronidase, it seemed desirable to reinvestigate this problem and to attempt to determine the relative significance of the hyaluronic acid capsule and the M protein in the virulence of group A streptococci.

distinct difference in the amount of the M substance elaborated by the glossy and matt variants of each pair of strains is apparent. Dilutions as high as 1:32 of the extracts of the matt variants showed positive reactions, whereas the undiluted extracts of the glossy variants gave only weak or no precipitin reactions.

For the mouse virulence tests, tenfold serial dilutions were prepared from samples removed from the cultures tested for M substance. The results of these tests are summarized in Table II. A marked difference in mouse virulence between the glossy and matt strains was found in spite of the fact

TABLE I

*Production of Type-Specific M Antigen by Encapsulated Variants of Group A Streptococci
Precipitin Reactions with M Extracts and Homologous Antisera*

Variant.....	Glossy					Matt				
Strain.....	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type....	1	3	12	14	19	1	3	12	14	19
Titration of M extract										
1:1	±	±	±	0	±	+++	+++±	+++	+++	+++
1:2	0	0	±	0	0	+++	++	+++	+++	+++
1:4	0	0	0	0	0	++	++	+++	++±	+++
1:8	0	0	0	0	0	++	++	++±	++±	+++
1:16	0	0	0	0	0	++	±	++±	++±	++
1:32	0	0	0	0	0	+	±	±	+	+
1:64	0	0	0	0	0	0	0	0	0	0

In titration of M antigen, degree of precipitation is indicated by a ++++ to ± scale; 0 represents no precipitation.

that each variant elaborated equally good hyaluronic acid capsules. All of the encapsulated glossy variants were relatively mouse-avirulent; the encapsulated matt variants were highly virulent, as indicated by death in 3 to 4 days occurring in mice infected with 10^{-8} cc. of culture, containing 2 to 3 microorganisms. Examination of selected mice that died all revealed encapsulated streptococci in the peritoneal exudate or heart blood. In additional experiments it was shown that polymorphonuclear neutrophils and monocytes in the exudate, obtained from the peritoneal cavity of mice inoculated with the encapsulated glossy variants, were actively phagocytizing the streptococci, but only rarely were the encapsulated matt variants taken up by the phagocytic leukocytes.

While these experiments clearly demonstrate the relationship of the M protein to mouse virulence, they do not bring out any evidence that the hyaluronic acid capsule has an effect in enhancing the virulence of these

TABLE II
Mouse Virulence of Encapsulated Variants of Group A Streptococci

Variant	Glossy					Matt				
Strain	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type	1	3	12	14	19	1	3	12	14	19
Dose cc.										
10^{-1}	D1	D1	D1	D1	D1	D1	D1	D1	D1	D1
	D1	D1	D2	D1	D1	D1	D1	D1	D1	D1
	D2	D1	D2	D2	D1	D1	D1	D1	D1	D1
10^{-2}	S	D2	S	D1	D1	D1	D1	D1	D1	D1
	S	D2	S	D2	D1	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
10^{-3}	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
10^{-4}	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D2
	S	S	S	S	S	D1	D1	D1	D1	D2
10^{-5}	S	S	S	S	S	D1	D1	D2	D1	D2
	S	S	S	S	S	D1	D1	D2	D2	D2
	S	S	S	S	S	D1	D1	D2	D2	D2
10^{-6}	S	S	S	S	S	D2	D2	D2	D2	D2
	S	S	S	S	S	D2	D2	D2	D2	D3
	S	S	S	S	S	D3	D2	D2	D2	D3
10^{-7}	S	S	S	S	S	D2	D2	D2	D2	D3
	S	S	S	S	S	D3	D2	D2	D3	D3
	S	S	S	S	S	D3	D3	D3	D2	S
10^{-8}	S	S	S	S	S	D3	D3	D3	D3	D3
	S	S	S	S	S	D3	D4	D3	D3	D3
	S	S	S	S	S	D3	D4	D3	S	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

streptococci for mice. Subsequent studies, however, with special techniques, revealed that the capsular material does have some influence on the pathogenicity of these bacteria.

Antiphagocytic Effect of the M Substance and Hyaluronic Acid Capsule.
 In order to analyze the method of destruction of these streptococci in the

animal body, an *in vitro* test was employed, which involved phagocytosis of the streptococci by the leukocytes of normal human blood. Experiments with mouse and human blood revealed that the streptococci were phagocyted in the blood of both species. Although it was theoretically preferable to use mouse blood as a test system since mouse virulence was being studied, it was found necessary to employ human blood because of the difficulty of obtaining sufficient quantities of blood from mice. Furthermore, the use of human blood was in itself of interest because of the relationship of the virulence of group A streptococci to human infection. Therefore, normal children's blood, which contains no detectable natural opsonins for these microorganisms, was employed.

The crude bovine testicular extract used in all the experiments as a source of hyaluronidase was found, in agreement with earlier workers, not to impair the phagocytic function of the leukocytes. Moreover, this enzyme prevents the formation of capsules of group A and group C streptococci during their growth cycle (Figs. 11 and 12). The question arose as to whether this crude tissue extract might contain proteolytic enzyme capable of digesting the M antigen of the streptococcal cell. Experiments were devised to test this possibility.

Washed group A streptococcal cells, strain S23, matt, type 14, from 500 cc. of Todd-Hewitt broth culture, were suspended in 4 cc. of physiological saline and heat-killed at 56°C. for 30 minutes. To one-half of these cells was added 2 cc. of crude bovine testicular extract containing 200 viscosity-reducing units of hyaluronidase; to the other half was added the same amount of extract inactivated by heating for 30 minutes at 60°C. After incubation of these mixtures at 37°C. for 18 hours, M extracts were prepared, diluted in a twofold serial manner, and tested against homologous antiserum.

Each test sample revealed approximately the same amount of M substance on titration. The same experiment was repeated with the addition to the testicular extract of a reducing substance, thioglycollic acid, in a final concentration of 0.01 M, in order to activate any previously inactive proteolytic enzyme which might be present. Again the M titres obtained were essentially the same in the two systems. In an additional experiment, using M substance in solution, samples of M extract were mixed with an equal volume of testicular extract containing 20 viscosity-reducing units of hyaluronidase or with heat-inactivated testicular extract, and incubated at 37°C. for 18 hours. Twofold serial dilutions of the mixtures were made and tested against homologous antiserum. No evidence of digestion of M substance was obtained. Further studies also revealed that the crude bovine testicular extract failed to digest gelatin or casein. Since the testicular hyaluronidase employed did not digest the M protein on the streptococcal cell or in solution, this extract was considered suitable as a source of hyaluronidase free of proteolytic enzyme.

To determine the antiphagocytic effect of the M substance and hyaluronic acid capsule, opsonic index experiments were performed.

Suspensions of well encapsulated, 8 hour cultures of the glossy and matt variants were turbidimetrically standardized, and blood agar plates were poured to estimate the bacterial count. To each of a series of tubes were added 0.05 cc. of culture dilution containing approximately 1,000,000 streptococci, 0.25 cc. of fresh, heparinized, human blood, and 0.05 cc. of hyaluronidase (10 viscosity-reducing units), type-specific anti-M serum, or saline. After 20 minutes in a rotating machine at 37°C., the tubes were plunged into ice water to stop phagocytosis, and smears were made and stained with Wright-Giemsa solution. The percent of active leukocytes and the number of cocci ingested by 100 polymorphonuclear neutrophils were determined.

TABLE III

Phagocytic Action of Normal Human Blood on Encapsulated and Decapsulated Variants of Group A Streptococci

Variant	Glossy					Matt									
Strain	CO	CT	ACT	SC	TH	CO	CT	ACT	SC	TH	CO	CT	ACT	SC	TH
Streptococcal type	1	7	11	4	9	1	7	11	4	9	1	7	11	4	9
Antibody	None					None					Anti-M Serum				
Encapsulated streptococci															
No. of cocci phagocytized by 100 leukocytes	1590	970	910	1560	2060	20	20	40	40	40	1210	1560	1510	1240	1760
Percentage of active leukocytes	65	96	61	64	64	1	1	1	1	10	65	66	64	60	89
Streptococci, unencapsulated with hyaluronidase															
No. of cocci phagocytized by 100 leukocytes	2760	2350	2240	2210	2640	130	210	210	570	515	2340	2410	1780	1700	2710
Percentage of active leukocytes	66	61	65	62	96	11	24	20	11	21	65	67	65	61	93

The results of the foregoing experiment are illustrated in Table III. The antiphagocytic properties of the M substance are shown by the fact that the matt, M-producing variants of both encapsulated and decapsulated streptococci are markedly more resistant to phagocytosis than the glossy non-M-producing variants. On addition of anti-M serum the encapsulated matt variants become as susceptible to phagocytosis as the encapsulated glossy variants. It will be noted that when hyaluronidase is added to the systems, with resulting removal of the capsules, more of the streptococci are phagocytized than in comparable systems in which the streptococci remain encapsulated because no hyaluronidase is added. These observations suggest that the hyaluronic acid capsule has some antiphagocytic property.

Photomicrographs of representative fields of smears made in the phagocytic experiments are shown for the strain S23, type 14 system. It should be noted that no phagocytosis occurs if M protein is synthesized by the streptococci regardless of whether the capsule is present (Figs. 13 and 14); however, it can be seen (Figs. 15 and 16) that phagocytosis of encapsulated, non-M-containing, glossy variants does take place, but that the decapsulated glossy variants are phagocytosed to a somewhat greater extent. As can be noted in Fig. 15, after phagocytosis of encapsulated organisms the capsule is no longer visible, which suggested that the leukocytes may contain hyaluronidase capable of digesting the capsular material. However, efforts to isolate this enzyme from large amounts of leukocytes obtained from human blood were unsuccessful.

Because the ultimate fate of the streptococci ingested by the phagocytes is not shown by opsonic index experiments such as that just described, a bacteriostatic test was employed. By this method it has been demonstrated that the ingested bacterial cells are destroyed by the phagocytic leukocytes (16).

Tenfold serial dilutions were used of a 3 hour broth culture of well encapsulated streptococci, ranging from 10^{-1} through 10^{-8} . In these experiments, 200 to 300 bacterial cells were contained in the 10^{-8} dilutions. To each of a series of tubes were added 0.05 cc. of culture dilution, 0.05 cc. of hyaluronidase (10 viscosity-reducing units), saline, or type-specific anti-M serum, and 0.25 cc. of fresh, heparinized, human blood. After incubation at 37°C . in a rotator for 3 hours, samples from each mixture were removed and streaked on rabbit blood agar plates. The resulting growth after 18 to 24 hours' incubation at 37°C . was recorded on a +++ to 0 scale.

Typical experiments are shown in Table IV, which reveals that the encapsulated, glossy variants are extremely susceptible to phagocytosis and fail to survive in the blood; on the other hand, the matt variants are resistant. In the presence of homologous anti-M serum these matt variants become as susceptible as the glossy variants. When hyaluronidase is added, which keeps the streptococci from regenerating their capsules throughout the 3 hour experimental period, it can be noted that the decapsulated matt variants retain their resistance to the bacteriostatic action of the blood. The decapsulated as well as the encapsulated matt variants become as susceptible on inclusion of anti-M serum in the system as the glossy variants.

It appears from the findings of these *in vitro* experiments that the M substance is far more important than the hyaluronic acid capsule in causing the streptococci to resist the phagocytic and bacteriostatic effect of the blood, but there was a definite indication from the opsonic index experiments that removal of the capsule enhanced the phagocytic effect of the leukocytes.

Demonstration of Protective Capacity of Crude Bovine Testicular Hyaluronidase against Group C Streptococcal Infection in Mice.—Although the rôle played by

TABLE IV
Bacteriostatic Action of Normal Human Blood on Capsulated and Decapsulated Variants of Group A Streptococci

Variant	Glucose					Malt				
	TA	TA	HV7	RD	TP9	TA	HV7	RD	TP9	TA
Media										
Bacteriologic type	1	3	12	11	19	1	12	11	19	11
Antibody										Anti M serum
Capsulated streptococci										
Inhibition of culture	10 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 2	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 3	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 4	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 5	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
Inhibition of culture	10 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 2	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 3	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 4	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 5	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
Streptococci decapsulated with hyaluronidase										
Inhibition of culture	10 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 2	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 3	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 4	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1
	10 5	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1	1 1 1 1

In bacteriostatic action, degree of growth is indicated on a 1 to 5 scale, 1 being less than 10 colonies are represented by single numerals; 2 indicates no growth.

the M protein of the streptococcal cell in resisting the phagocytic action of leukocytes contained in human blood appeared clearly defined in the *in vitro* experiments, the effect of the capsule was still not well understood. We, therefore, attempted to study the problem in an *in vivo* system employing the mouse as a test animal.

To make certain that the preparation containing hyaluronidase was active in the *in vivo* system selected, the bovine testicular extract was tested for its effect in protecting mice against infection with group C streptococci. Previous workers, with one exception, obtained good protection in similar experiments.

The procedure employed was essentially the same as that described by Kass and Seastone (8) for protection experiments with group A streptococci. Mice were inoculated intraperitoneally with tenfold serial dilutions of 4 hour broth cultures of encapsulated group C streptococci, strain D181. The number of streptococci inoculated were calculated by pouring rabbit blood agar plates and the colony counts were 26 in the 10^{-7} dilution and 240 in the 10^{-6} dilution. Treatment with 0.5 cc. crude bovine testicular extract containing 100 viscosity-reducing units was started 2 hours later. Injections of enzyme were given intraperitoneally every 2 hours for the first 12 hours, every 4 hours during the next 36 hours, and every 12 hours for the last 48 hours; a total of 19 injections.

In Table V are illustrated the results of the protective influence of crude bovine testicular hyaluronidase on mice infected with group C streptococci. The enzyme protected all mice completely against 1,000 M.L.D. and some of the mice against even 100,000 M.L.D. A control group of mice treated with enzyme inactivated by heating to 60°C. for 30 minutes died as rapidly as the untreated virulence controls. These findings are remarkably similar to those of Hirst (4), who employed leech extract as a source of enzyme.

Method of Demonstrating Mouse Protection against Group A Streptococci with Hyaluronidase.—Since crude testicular hyaluronidase was thus found to be effective in protecting mice against infections with encapsulated group C streptococci (Table V), experiments were undertaken in an attempt to also obtain protection in mice with encapsulated group A streptococci. In the studies of Kass and Seastone (8), who were successful in such experiments, it was noted that the final dilutions of the streptococci were made in testicular extract and allowed to remain for 5 minutes at room temperature before injection of the mixture; streptococci used for the controls, however, were diluted in plain broth. Blundell (6) had previously shown that simultaneous injections of a mixture of bovine testicular extract and streptococci, followed by treatment with the enzyme, provided the greatest delay in the time of death as compared with untreated control mice. These observations suggested an explanation for the fact that we had earlier failed to obtain any protection of mice against group A streptococci since 2 hours had elapsed before enzyme treatment was started.

The effect of giving the hyaluronidase simultaneously with the streptococci

by diluting the bacteria in the testicular extract was compared with the effect of delaying treatment by diluting the streptococci in plain broth and giving the first injection of enzyme 2 hours later. The results of such an experiment are shown in Table VI. Encapsulated group A type 14 streptococci, strain S23, were employed. The colony count in the 10^{-7} dilution was 19 colonies and in the 10^{-8} dilution, 2 colonies. The treatment schedule was the same as that used against group C streptococci, except where earlier treatment was

TABLE V
*Demonstration of Protective Activity of Crude Testicular Hyaluronidase
Mouse Protection against Group C Streptococci*

Treatment of mice	Culture of streptococci, strain D151					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Unheated testicular hyaluronidase	D1	D2	S	S	S	S
	D2	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
Heated testicular hyaluronidase*			D1	D1	D1	D1
			D1	D1	D1	D2
			D1	D1	D2	D2
			D1	D2	D2	D2
Untreated virulence controls	D1	D1	D1	D1	D1	D1
	D1	D1	D1	D1	D1	D2
	D1	D1	D1	D2	D2	D2
	D1	D1	D1	D2	D2	D2

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

* Hyaluronidase heated at 60°C. for 30 minutes.

given by diluting the cultures in hyaluronidase. In diluting the streptococci in hyaluronidase, the concentration of enzyme was adjusted so that the mouse received the same amount of enzyme as that used in the later therapeutic doses; i.e., 100 viscosity-reducing units.

When the streptococci were diluted in plain broth and the mice subsequently treated after a lapse of 2 hours with 19 injections of hyaluronidase for 96 hours, only a slight delay in the rate of death was obtained as compared with the untreated controls. In contrast, when the streptococci were diluted in a solution of hyaluronidase, even though no additional treatment was given, there was some protection in the 10^{-5} dilution and a delay in the time of death in the other dilutions. Moreover, when the mice, in addition to the first dose

TABLE VI
Method of Demonstrating Protective Action of Crude Testicular Hyaluronidase against Group A Streptococci
Mouse Protection Test

Treatment of mice		Culture of streptococci, strain S23 matt, type 14				
		cc.				
		10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Streptococci diluted in plain broth	Untreated virulence controls	D1	D1	D1	D2	D3
		D1	D1	D1	D2	D3
		D1	D1	D1	D2	D3
		D1	D1	D2	D2	D3
		D1	D1	D2	D2	D3
	Treated with multiple injections of hyaluronidase	D1	D2	D2	D3	D4
		D1	D1	D2	D3	D3
		D1	D1	D2	D3	D3
		D1	D1	D2	D3	D4
		D1	D2	D3	D3	D4
Streptococci diluted in solution of hyaluronidase	No additional treatment	D2	D2	D3	D3	D4
		D2	D2	D3	D4	D5
		D2	D2	D3	D4	D5
		D2	D2	D3	D5	S
		D2	D2	D4	D5	S
	Treated with multiple injections of hyaluronidase	D2	D2	D4	D5	S
		D1	D1	D4	D5	S
		D1	D2	D5	S	S
		D1	D3	D5	S	S
		D2	D3	D6	S	S
		D2	D3	D6	S	S
		D2	D3	D6	S	S
		D2	D3	S	S	S
		D2	D3	S	S	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

Duplicate sets of mice treated with hyaluronidase heated at 60°C. for 30 minutes all died at the same rate as untreated virulence controls.

of hyaluronidase, were also treated with multiple injections (19 injections for 96 hours), protection was obtained against 10 M.L.D., with marked delay in the time of death of the other mice, as compared with the untreated virulence controls.

It is thus apparent that protection against group A streptococcal infection in mice can be obtained when hyaluronidase is given concomitantly with the

TABLE VII

Continued Action of Anesthetics and Single Testicular Hydronephrosis in Females of *Mus musculus* Infected with *Sem. 1. Strychnina*

Mouse Peritoneum Tests

Treatment of mice		Number of mice which survived 2 weeks, out of							
		25							
		100	100	100	100	100	100	100	100
Anesthetized alone	Single injection of anes-	11	13	6	6	6	6	6	6
	thetic given 12 hrs.	11	13	6	6	6	6	6	6
	before strychnine	11	10	6	6	6	6	6	6
		11	6	6	6	6	6	6	6
		11	6	6	6	6	6	6	6
Testicular hydra-	100 mice 3 injections of				13	13	11	6	
	nephrosis alone				13	13	6	6	
	hyaline case*				13	13	6	6	
					13	13	6	6	
					13	13	6	6	
Both anesthesia	Test given as above*	11	6	6	6	6			
	and testicular	11	6	6	6	6			
	hydronephrosis	11	6	6	6	6			
		11	6	6	6	6			
		11	6	6	6	6			
Control. Anes-	Anesthesia as above;	11	13	6	6				
	thetic alone	11	13	6	6				
	and saline	11	6	6	6				
	single instead of tri-	11	6	6	6				
	hydrate	11	6	6	6				
Unanesthetized alone		11	13	6	6	13	13	13	13
		11	13	6	6	13	13	13	13
		11	13	6	6	13	13	13	13
		11	13	6	6	13	13	13	13
		11	13	6	6	13	13	13	13

0 indicates survival of one mouse for at least 2 weeks.

1 with hyaline* indicates death of one mouse within that number of days.

* Strychnine used for induction of these mice were killed by hyaline case.

survived and followed by additional treatment with the anesthetic but not if the survivors are killed in breath and injected two mice followed 2 hours later by treatment with hyaline case.

Combined Effect of Anti-M Serum and Hyaluronidase in Mouse Protection against Group A Streptococci.—Having demonstrated a method by which some protection of mice against encapsulated group A streptococci can be obtained by using hyaluronidase, thus confirming the work of Kass and Seastone (8), the protective effect of type-specific anti-M serum was compared with that of hyaluronidase; and the combined effect of these 2 agents was also investigated.

The method of treatment, which was demonstrated as being most effective in the preceding experiment (Table VI), was used. Five groups of mice were selected and each treated in a different manner. The first group was injected intraperitoneally with 0.5 cc. type-specific antiserum 16 hours prior to intraperitoneal inoculation with the streptococci, and no further treatment was given. The second group was inoculated with streptococci diluted in hyaluronidase and these mice were further treated with multiple injections of this substance as in the preceding experiment (Table VI). The third group received the combined treatment of the first two groups; *i.e.*, the mice received antiserum, were inoculated with streptococci diluted in hyaluronidase, and were further treated with multiple injections of the enzyme. The fourth group, used as a control for the effect of repeated injections, was treated with antiserum as in the first group, and received multiple injections of saline instead of hyaluronidase after inoculation of streptococci. The fifth group served as untreated virulence controls.

Table VII summarizes the findings in this experiment. With a single injection of antiserum, protection against 10,000 to 100,000 M.L.D. of group A streptococci was obtained. Protection against 10 M.L.D. was noted with hyaluronidase. With the combination of antiserum and testicular hyaluronidase there was an additive effect affording protection against 1,000,000 M.L.D., as compared with the untreated virulence controls. The control group, given antiserum and multiple injections of saline instead of hyaluronidase, revealed that there was no non-specific protective effect from the multiple injections.

The results of this experiment show that hyaluronidase, effective against the capsular material, and type-specific anti-M serum, specifically directed against the M substance of the streptococcal cell, each has a protective effect against group A streptococcal infection in mice. These findings also illustrate that the anti-M serum protects against 1,000 to 10,000 times as many M.L.D. as hyaluronidase and that the combined action of these two substances provides greater protection for mice than either one alone.

DISCUSSION

The protective effect of hyaluronidase derived from various sources has usually been unquestioned with regard to experimental group C streptococcal infections in laboratory animals, but conflicting evidence has been obtained by different investigators as to its protective action in group A streptococcal infections in mice. This has led to different concepts as to the influence of the hyaluronic acid capsule on the virulence of group A streptococci. Those workers, notably Hirst (4), who failed to protect mice against group A streptococcal infections with hyaluronidase, concluded from their studies that,

unlike group C streptococci, other factors such as the M antigen were more significant than the capsule in determining the virulence and invasiveness of group A streptococci.

The present work provides an explanation for the discrepancy between this point of view and that of Kass and Seastone (8) who, on finding some protection against group A streptococcal infection in mice treated intensively with hyaluronidase, expressed the view that the capsules of both group A and group C streptococci are responsible for the greater part of their virulence. Furthermore, these workers believed that the M antigen might be of only minor importance, since group A streptococci may elaborate M substance without necessarily being virulent for mice.

The evidence obtained in this study brings out the fact, well recognized by all investigators, that no single cellular component or product of group A streptococci, or indeed probably of any microorganism, contributes exclusively to the property of virulence. It is also recognized that factors still not known are essential to the development of virulence for different animal species. As an illustration, M-containing, encapsulated variants freshly isolated from patients, although evidently virulent for man are usually avirulent for mice. Apparently other cellular functions in addition to the production of capsular polysaccharide or M protein are also involved in the virulence of these microorganisms for mice. However, unless the strain elaborates the M antigen, it is not virulent for either mouse or man. Of the known factors, both the hyaluronic acid capsule and the M antigen of the streptococcal cell have been shown to contribute to the exhibition of virulence, and correspondingly the agents which lead to their destruction contribute to the protection of mice against infections with these streptococci. Thus, it has been amply demonstrated that the matt variants which elaborate the M substance are virulent for mice and resist phagocytosis; the glossy variants, however, which produce little or no M substance, are avirulent for mice and are susceptible to phagocytosis, even though both variants are encapsulated. This offers substantial evidence that the M substance is an important factor in the pathogenicity of group A streptococci.

In phagocytic experiments, on comparing the encapsulated streptococci and streptococci decapsulated with hyaluronidase, it was noted that the decapsulated bacteria were taken up by the leukocytes more readily and in larger numbers than the encapsulated forms. Further evidence was provided that the capsule is actually concerned with virulence to a certain extent by demonstrating that mice were protected against 10 M.L.D. of group A streptococci by hyaluronidase treatment.

To obtain this protective effect, it was essential to have the capsules removed by diluting the streptococci in a solution of hyaluronidase before injection and further to prevent capsule regeneration by frequent and continued

treatment with enzyme. No protection was obtained if the streptococci were diluted in broth and the multiple injections of hyaluronidase started 2 hours later, because the injected streptococci had multiplied so many times during the 2 hour interval that the enzyme therapy was inadequate to permit the host to deal with the increased number of bacteria. The technique of simultaneous injection of enzyme and streptococci explained the success of Kass and Seastone (8) in obtaining protection in mice; and it is likely that other workers also would have shown protective effects if such early and intensive treatment had been carried out.

The protection of mice could be increased from the 10 M.L.D. obtained by enzyme therapy alone to 1,000,000 M.L.D. by the combined use of anti-M serum and enzyme. A single injection of 0.5 cc. of antiserum 16 hours before inoculation of mice was sufficient to afford almost this much protection; and previous work indicates that even this dose of serum was a great excess (17). The greater protection afforded by the anti-M serum emphasizes the importance of the M substance in virulence of group A streptococci and is in agreement with other studies in this report dealing with the opsonic index, bacteriostatic, and mouse virulence experiments.

As previously demonstrated by Ward and Lyons (18) and shown in this report, encapsulation does not necessarily exclude phagocytosis. In the present experiments, decapsulated streptococci were slightly more susceptible to phagocytosis than those which retained their capsules. The encapsulated variants were nevertheless still highly susceptible to phagocytosis unless in addition to capsular substance they elaborated M antigen, whereas M-producing variants were only phagocytosed to a very slight extent even though they were decapsulated with hyaluronidase. This suggests that encapsulation of group A streptococci and resistance to phagocytosis do not bear a direct relation to each other.

Though it is hazardous to generalize from these experiments in mice that the M substance and the capsular material play similar rôles in infections in man, the natural host for group A streptococci, it is worthy of note that one test system employed was dependent upon the phagocytic leukocytes in normal human blood. Both the M substance and the capsules had anti-phagocytic properties against these human leukocytes. That the M protein is intimately related to virulence of streptococci for man is further supported by the fact that in this laboratory no strain tested and shown to lack M antigen has ever been isolated from a patient in the acute phase of streptococcal infection. In all strains tested, whenever the M substance could not be identified with the available type-specific antisera, it was possible by immunizing rabbits to show that the strain produced some other M substance not previously identified in this laboratory. On the other hand, strains have been isolated from patients in the acute phase of their infection which lacked

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EXPLANATION OF PLATES

These photographs were made by Mr. Joseph B. Haulenbeek.

PLATE 13

All preparations made with India ink; 3½ hour broth cultures of group A streptococci employed. × 1500.

FIG. 1. Capsule of glossy variant of type 1 (strain T1).

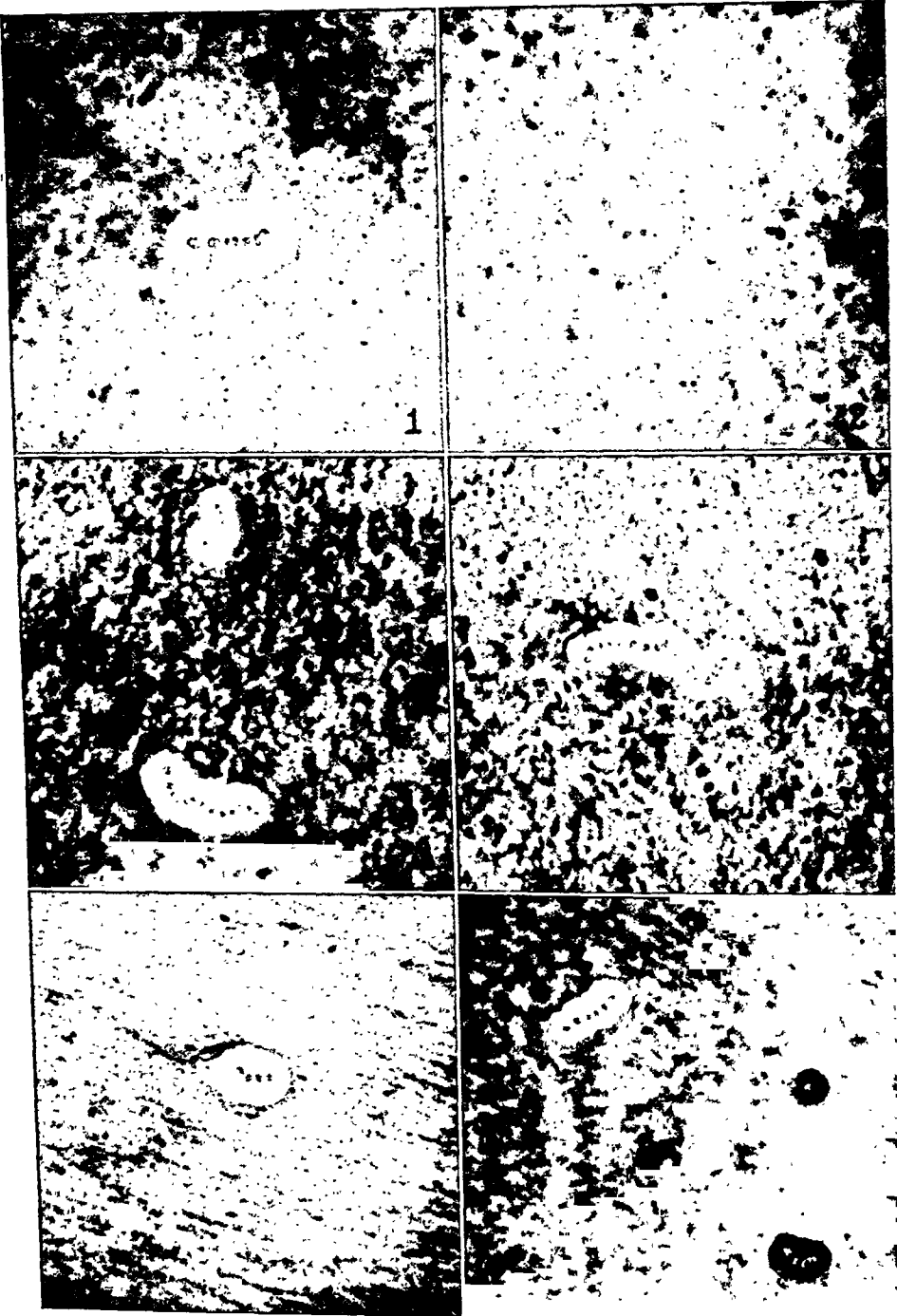
FIG. 2. Capsule of matt variant of type 1 (strain T1).

FIG. 3. Capsule of glossy variant of type 3 (strain T3).

FIG. 4. Capsule of matt variant of type 3 (strain T3).

FIG. 5. Capsule of glossy variant of type 12 (strain NY5).

FIG. 6. Capsule of matt variant of type 12 (strain NY5).



(Rothbard: Group A streptococcus infections)

PLATE 14

FIG. 7. Capsule of glossy variant of type 14 (strain S23).

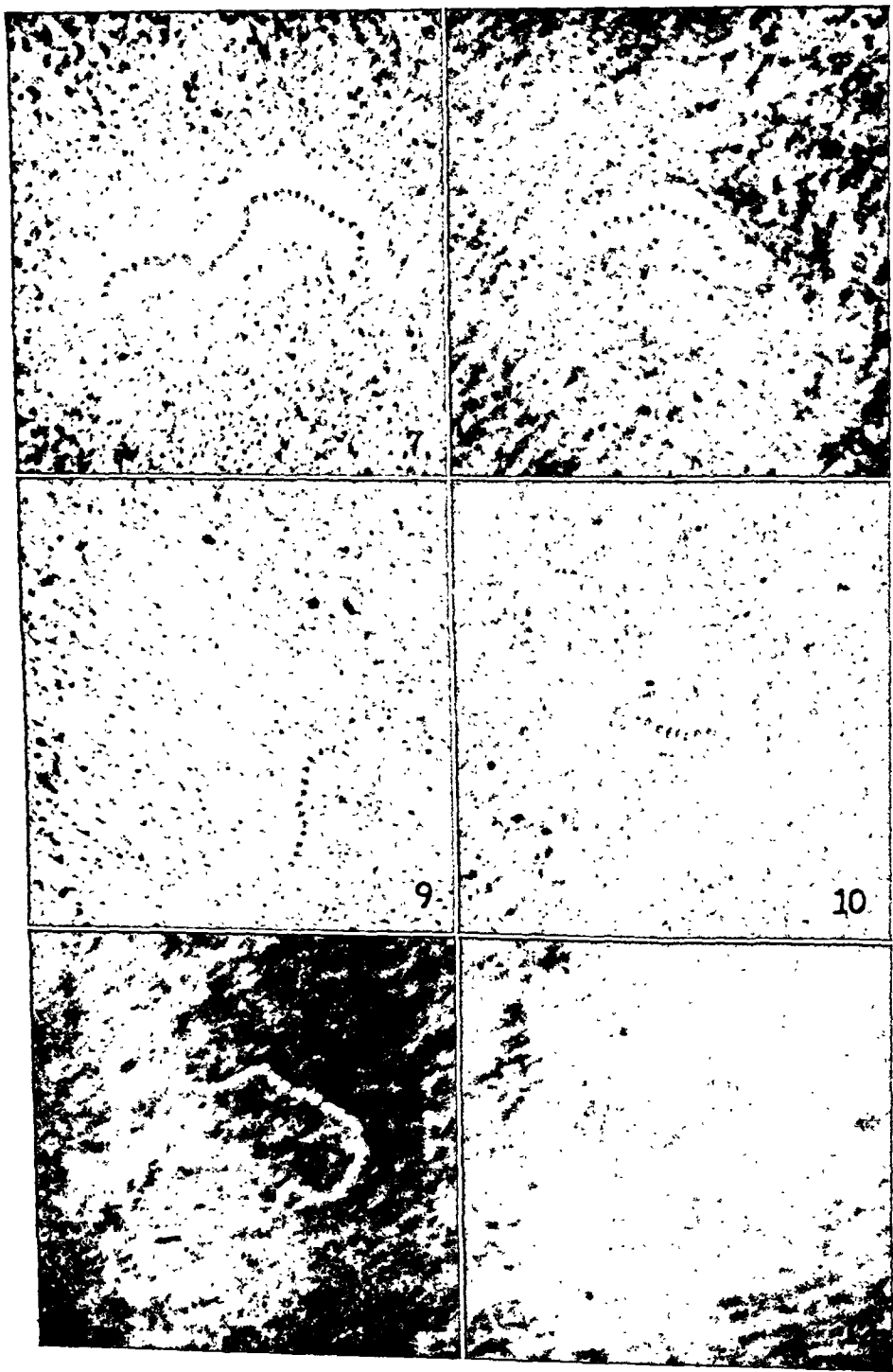
FIG. 8. Capsule of matt variant of type 14 (strain S23).

FIG. 9. Capsule of glossy variant of type 19 (strain T19).

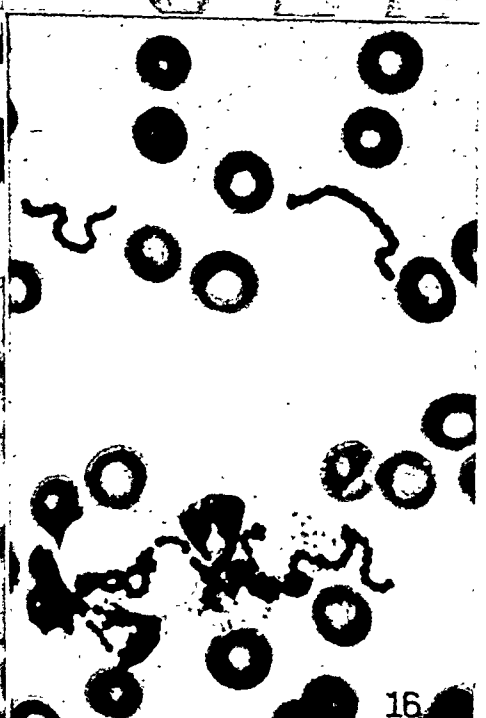
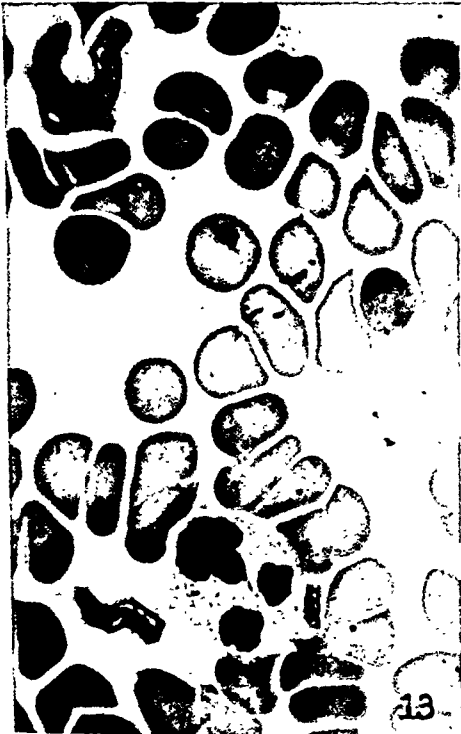
FIG. 10. Capsule of matt variant of type 19 (strain T19).

FIG. 11. Decapsulated glossy variant of type 14 (strain S23); 3 minutes after addition of bovine testicular extract.

FIG. 12. Decapsulated matt variant of type 14 (strain S23); 3 minutes after addition of bovine testicular extract.



(Rothbard: Group A streptococcus infections)



INFLUENZA VIRUS INFECTION IN THE HAMSTER*

A STUDY OF INAPPARENT VIRUS INFECTION AND VIRUS ADAPTATION

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Viruses may multiply in a host without producing manifest illness or gross evidence of a pathological process. It is well known, for example, that inapparent or subclinical infections commonly occur in such virus diseases of man as mumps, influenza, and poliomyelitis. Infections of this sort often assume great importance in the epidemiology of a virus disease and in calling forth immunity in the individual. One of the most interesting examples of inapparent virus infection is seen in the process of adapting a virus to a new host by serial blind passage. Infection may not be apparent during repeated passages until the virus, presumably as a result of variation, acquires the capacity to produce lesions in the host. Although the method of serial passage has been used extensively in adapting viruses to experimental animals, or in altering the properties of a virus, little is known regarding the fundamental mechanism of this process.

The hamster has been generally regarded as having only limited susceptibility to influenza virus infection. Although intranasal inoculation of influenza virus calls forth specific serum antibodies, lung lesions usually have not been observed even after repeated passage (1). The immune response of hamsters has been used as an indirect method for detecting influenza virus in throat washings (2) and it has been reported that the hamster is as effective as the ferret for this purpose (3). The present paper describes further studies on the behavior of influenza virus in the hamster, undertaken in an attempt to learn more about the factors involved in an inapparent virus infection and in the adaptation of a virus to a new host.

Methods

Virus Strains.—Two strains of influenza A virus (PR8 and Ga. 47) and one strain of influenza B virus (Lee) were used in the present study. The Ga. 47 strain was isolated from a patient in Atlanta during the influenza A epidemic of 1947 by intraamniotic inoculation of a throat washing into chick embryos. It was subsequently maintained by allantoic passage in 11-day-old embryos.

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carried through eleven serial passages as described under Methods. The original virus suspension agglutinated red cells in high titer, fixed complement in dilution of 1:160, and had mouse mortality and egg infectivity titers of $10^{-4.5}$ and $10^{-8.6}$ respectively.

The results obtained with this virus strain are summarized in Table I. During the first six passages, no lung lesions or fatalities were observed in the

TABLE I
Serial Passage of Ga. 47 Influenza Virus in Hamsters

Passage No.	Tests for virus in hamster lung extracts*						Pathogenicity of virus in hamsters†	
	Titration in		Complement fixation titer	Hemagglutination titer Red cells				
	Eggs	Mice		Chicken	Guinea pig	Human	Lung lesions	Mortality
0§	8.6	4.5	160	512	1024	1024		
1	6.2	2.5	80	—	—	—	0, 0	0/2
2	4.8	1.5	80	—	—	—	0, 0	0/2
3	6.1	1.3	80	—	—	—		
4	5.4	1.3	80	—	—	—	0, 0	0/2
5	6.6	3.2	80	—	—	—	0, 0	0/2
6	6.0	4.5	640	—	—	—	±, ±	0/2
7	7.5	4.6	320	—	20	160	+++++, +++++	2/2
8	7.2	5.3	640	—	640	320	+++++, +++++	2/2
9	7.0	4.6	640	—	160	320	+++++, +++++	2/2
10	8.5	5.6	640	—	320	1280	+++++, +++++	2/2
11	8.5	5.6	640	80	1280	1280	+++++, +++++	2/2

* Titers expressed as the reciprocal of the dilution end-points (see Methods). — = less than 1:20.

† Two hamsters in each passage observed for 10 days following inoculation.

Lung lesions recorded as relative degrees of consolidation of total lung. ++++ = complete consolidation.

Under mortality, numerators indicate the number of hamsters dying within 10 days after inoculation, and the denominators the number of hamsters inoculated.

§ Allantoic fluid suspension of the Ga. 47 strain.

hamsters followed for a period of 10 days. Nevertheless, virus was present in high titer as shown by egg infectivity tests with extracts of the lungs obtained 3 to 4 days after inoculation. Virus was also demonstrable in the extracts by mouse mortality tests, but in lower titer. It will be seen that the total amount of virus present in the lungs as determined by titration in eggs and mice decreased during the first two passages. By the sixth passage, however, the mouse mortality titer was comparable to that of the original virus suspension, while the egg infectivity titer was $10^{-6.0}$ as compared with the original titer of $10^{-8.6}$. Complement-fixing antigen was present in extracts

of the lungs of all the passages, but showed a sharp increase on the sixth passage. The extracts during these early passages failed to cause agglutination of chicken, guinea pig, or human red blood cells. Occasionally a weak agglutination of human or guinea pig red blood cells was observed with a 1:20 dilution of the lung extracts, but the reaction was too indefinite to record.

On the seventh hamster passage a sudden change in the course of events was observed. Extensive pulmonary consolidation occurred and the hamsters died on the 6th day after inoculation. The most striking finding in the tests for virus in the lungs was the appearance of agglutinins for human and guinea pig red cells for the first time. The egg infectivity titer increased from $10^{-6.0}$ to $10^{-7.5}$, but no change in mouse mortality titer was observed. The latter, together with the titer of complement-fixing antigen, had increased during the preceding passage.

The lethal character of the virus persisted on subsequent passages. Agglutination of chicken red blood cells, however, was not observed until the eleventh passage. A further increase in the total amount of virus in the lungs, as determined by titration in mice and eggs, occurred on the tenth passage. The content of complement-fixing antigen remained constant at a titer of 1:640. The lung extract of the thirteenth passage (not shown in the table) produced complete pulmonary consolidation and death in hamsters in dilutions through $10^{-6.0}$.

2. PR8 Strain.—In the next experiment the PR8 strain of influenza A virus was inoculated intranasally into hamsters and carried through twelve serial passages. The virus suspension used for inoculation consisted of a freshly prepared PR8 mouse lung extract at a concentration of 10 per cent.

Table II shows the results of this experiment. Infectivity tests in eggs were not carried out in this experiment. In general, the results are similar to those obtained with the Ga. 47 strain (Table I). Lung lesions were not observed during the first six passages and extracts of the lungs failed to agglutinate red blood cells. Nevertheless, virus was demonstrable in all of the passages by mouse mortality tests and also by means of the complement fixation test. On the seventh hamster passage a fatal disease with pulmonary consolidation was produced, together with the appearance of hemagglutinins in extracts of the lungs for guinea pig and human red cells, but not for chicken red cells. At this time the mouse mortality and complement fixation titers of the lung extracts increased. On the eighth and ninth passages, however, the hamsters did not die and lung lesions were not observed. Extracts of the lungs then failed to agglutinate guinea pig red cells and showed a decreased capacity to agglutinate human red blood cells. During these passages the mouse mortality titer decreased, but no change in the titer of complement-fixing antigen occurred. By the tenth passage a fatal disease was again produced and on the eleventh passage, hemagglutinins for chicken, guinea pig, and human red blood

cells were present in high titer. A sharp increase in the mouse mortality titer occurred on the eleventh passage, coinciding with the first appearance of agglutinins for chicken red cells.

As in the preceding experiment, a close correlation between the capacity of the extracts to produce lung lesions in the hamster and hemagglutination of human and guinea pig cells was evident. In both experiments lung lesions and a fatal disease appeared suddenly on the seventh hamster passage.¹ With the PR8 strain, for some unknown reason, the lethal character of the virus

TABLE II
Serial Passage of PR8 Influenza Virus in Hamsters

Passage No.	Tests for virus in hamster lung extracts					Pathogenicity of virus in hamsters	
	Mouse mortality titer	Complement fixation titer	Hemagglutination titer Red cells			Lung lesions	Mortality
			Chicken	Guinea pig	Human		
0* ML	6.5	320	1280	640	640		
1	4.0	20	—	—	—	0, ±	0/2
2	3.5	80	—	—	—		
3	3.3	80	—	—	—	0, 0	0/2
4	3.6	40	—	—	—	0, 0	0/2
5	3.6	80	—	—	—	0, 0	0/2
6	4.0	80	—	—	—	0, ±	0/2
7	4.5	320	—	160	640	++++, +++++	2/2
8	4.3	320	—	—	160	0, 0	0/2
9	2.3	320	—	—	40	0, +	0/2
10	3.0	320	—	—	—	++++, +	1/2
11	6.3	640	320	640	640	++++, +++++	2/2
12	6.5	640	1280	1280	1280	++++, +++++	2/2

* 10 per cent PR8 mouse lung extract.

See footnotes under Table I.

was lost on the eighth and ninth passages but reappeared on the tenth passage. An unusual opportunity was thus afforded for correlating the pathogenicity of the virus with its other properties.

3. *Lee Virus*.—For comparison with the results obtained in the preceding experiments with influenza A viruses, the Lee strain of influenza B virus was selected for a passage series in the hamster. A freshly prepared allantoic fluid suspension of Lee virus was inoculated intranasally into hamsters and extracts of the lungs were passed serially at 2 day intervals.

¹ In order to rule out a bacterial component in the production of the lung lesions, cultures of the lung extracts were taken throughout these experiments. Except for occasional contaminants, the cultures were negative.

The results are summarized in Table III. The course of events differed markedly from that observed with the influenza A virus strains (Tables I and II). Lung lesions and fatalities occurred with the first passage and persisted through the series of eleven passages. There was no apparent increase, however, in the capacity of the virus to produce pulmonary lesions on repeated passage in the hamster. Extracts of the lungs from all passages caused agglutination of chicken, human, and guinea pig red cells. The mouse infectivity titer and complement fixation titer of the lung extracts remained re-

TABLE III
Serial Passage of Lee Influenza Virus in Hamsters

Passage No.	Tests for virus in hamster lung extracts						Pathogenicity of virus in hamsters	
	Titration in		Complement fixation titer	Hemagglutination titer Red cells			Lung lesions	Mortality
	Eggs	Mice		Chicken	Guinea pig	Human		
0*	6.5	3.6	150	512	128	512		
1	5.6	3.3	320	640	640	1280	-----, =	1.2
2	4.3	3.0	160	40	320	160	-----, ---	1.2
3	6.3	3.6	160	160	160	320	---, C	0.2
4	5.3	4.5	640	160	640	160	-----, ---	1.2
5	4.6	3.5	320	160	320	320	-----, C	1.2
6	6.3	2.6	320	40	160	160	---, C	0.2
7	3.5	3.3	640	30	160	30	-----, ---	1.2
8	4.5	3.5	320	30	160	320	-----, -----	1.2
9	5.0	3.6	640	160	320	320	-----, -	1.2
10	4.0	3.3	320	640	640	640	---, ---	0.2
11	5.4	3.5	320	160	320	320	-----, -----	2.2

* Allantoic fluid suspension of Lee influenza virus.

See footnotes under Table I.

markably constant throughout, differing little from the titers of the original virus suspension used for inoculation.

Effect of Incubation at 37°C. on Chicken Red Cell Agglutination with Hamster Lung Extracts.—The preceding experiments demonstrated that influenza A virus in hamster lung extracts from the early passages failed to agglutinate red blood cells, even though virus was readily demonstrable by infectivity or complement fixation tests. After six serial passages, however, agglutinins for human and guinea pig red cells appeared in high titer, but agglutinins for chicken red cells either did not occur or produced only an indefinite reaction until the eleventh passage. This finding is of interest in view of the observation of Burnet and Bull (8) that freshly isolated strains of influenza virus in the chick embryo agglutinate mammalian red cells in higher titer than fowl

red cells. A further study of this phenomenon in extracts of hamster lungs was therefore undertaken.

The hamster lung extracts of the sixth, seventh, tenth, and eleventh passages of the Ga. 47 strain of influenza virus (see Table I) were selected for study. Portions of the 10 per cent saline extracts, which had been previously cleared by low speed centrifugation, were spun at about 12,000 R.P.M. for 30 minutes to sediment the virus. The supernatant fluids were discarded and the sediments were resuspended in the original volume of 0.9 per cent saline. The suspensions were then placed in a 37°C. water bath for 6 hours. Following incubation, the suspensions thus treated and portions of the original extracts kept at 4°C. were tested for capacity to agglutinate chicken red cells. The end-point was recorded as the highest dilution of the extract that produced complete agglutination of the red cells.

Table IV shows the results of these tests. It will be seen that the procedure of high speed centrifugation and incubation at 37°C.² was effective in bringing

TABLE IV

Effect of Incubation at 37°C. on the Hemagglutination Titer of Hamster Lung Extracts

Hamster passage No.	Agglutination titer of lung extracts with chicken red cells	
	Before incubation	After incubation at 37°C. for 6 hrs.*
6	<20	<20
7	<20	40
10	<20	640
11	80	640

* Extracts spun at 12,000 R.P.M. for 30 minutes, sediment resuspended in original volume of 0.9 per cent saline, then incubated at 37°C. for 6 hours (see text).

out agglutinins for chicken red cells in those extracts which originally caused agglutination of only human and guinea pig red cells (compare Table I). Furthermore, the titers of chicken red cell agglutinins in the extracts thus treated were approximately the same as the titers of human and guinea pig red cell agglutinins obtained with the untreated extracts. In additional tests (not shown in the table) it was found that the titers of agglutinins for the mammalian types of red cells were not increased by the method of centrifugation and incubation at 37°C. The implications of these findings will be considered in the discussion.

Effect of Influenza Virus Infection on the Temperature of the Hamster.—During the serial passages of the influenza A virus strains already described, rectal temperatures were taken daily on the hamsters during the 10 day observation period. It was found that a precipitous drop in temperature oc-

² Incubation of the extracts at 37°C. for 6 hours without preliminary centrifugation was also effective in calling forth chicken cell agglutinins. The titers obtained, however, were not as high nor was the reaction as definitive following centrifugation as outlined.

curred on the 2nd to 4th day after inoculation of either the Ga. 47 or PR strains of virus. During the early passages of these viruses, before lung lesions were produced, this temperature response was observed in only about half of the inoculated hamsters and the temperatures usually returned to normal

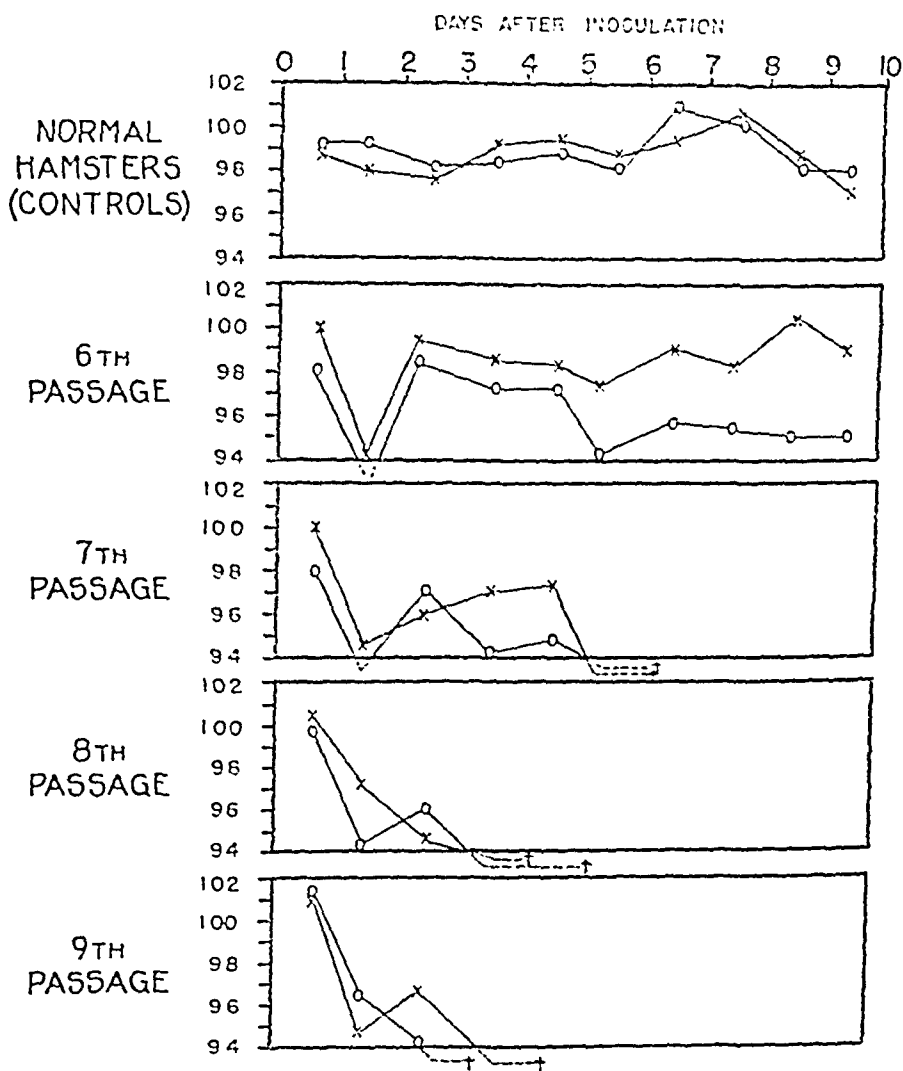


CHART 1. Temperature response in hamsters to Ga. 47 influenza virus.

levels within 24 to 48 hours. After the sixth passage, however, the drop in temperature occurred consistently and subnormal levels persisted until death.

The temperatures of the hamsters inoculated with the Ga. 47 strain from the sixth to the ninth passage are shown in Chart 1. The temperatures of two normal hamsters are included as controls. Temperatures less than 94°F. are indicated by dotted lines, since the thermometers used did not record below

this level. On the sixth passage the temperatures of both hamsters dropped sharply to 94°F. or less 24 hours after inoculation, but returned to normal limits within 48 hours. In one hamster the temperature then remained normal, while in the other it gradually fell to subnormal levels during the 10 day period. The hamsters in this passage did not die and only minimal lung lesions were observed (see Table I). In the subsequent passages the temperature either remained low or showed a rise followed by a second drop to less than 94°F. It will be noted also that the interval between inoculation and death decreased with passage.

DISCUSSION

The course of events following the inoculation of influenza virus into hamsters herein reported may represent an unusual instance of virus activity in a new host. Regardless of whether this is the case or not, the findings shed some light on the behavior of a virus during inapparent infection and on the process whereby a virus assumes new characteristics as a result of serial passage. First of all, it is clear that the influenza A strains used in these experiments multiplied readily in the hamster lung. Although illness and pulmonary lesions were not observed in the early passages, virus was demonstrable from the start in extracts of the lungs by infectivity tests in mice and in embryonated eggs, or by means of the complement fixation test. The findings are similar to those of Hirst (9) who recently reported that maximum multiplication of virus occurs in mouse lungs following the inoculation of egg-adapted influenza A virus strains, before lung lesions are produced as a result of serial passage. These results emphasize the fact that no correlation may exist between the pathogenic potentialities of a virus and its capacity to multiply in a host. If the conditions are such that the virus, though actively multiplying in the host, fails to acquire pathogenic qualities, even after repeated passage, the infection might be overlooked. Obviously, such findings have practical implications regarding the isolation of viruses in experimental animals.

In the present experiments the inapparent virus infection in the hamster, which persisted for six serial passages, changed dramatically to a severe disease characterized by extensive pulmonary consolidation and death. The various tests utilized for studying the content of virus in the lungs during passage revealed certain findings of interest, which accompanied the abrupt change in pathogenicity of the virus. Agglutinins for human and guinea pig red blood cells appeared and a marked increase occurred in the amount of complement-fixing antigen. Although the tests for infectious virus are less accurate and more difficult to interpret, there was a definite tendency toward an increase in the total amount of virus in the lungs. In both experiments with influenza A strains the titer of virus in the lungs after eleven serial passages was as high or higher than the titer of the original inoculum. The findings as a whole

1. Two influenza A strains (Ga. 47 and PR8) multiplied readily in the hamster lung, although no lung lesions were produced during six serial passages. On further passage both viruses abruptly acquired the capacity to produce pulmonary consolidation and death of the animals.

2. Extracts of the lungs during the early passages contained complement-fixing antigen and infectious virus, as revealed by titration in mice and embryonated eggs. Agglutinins for chicken, human, and guinea pig red cells, however, were not demonstrable at this time. With further passage a close correlation was observed between the capacity of the virus to produce lung lesions in the hamster and to agglutinate mammalian types of red cells. In addition, quantitative changes in the virus population were demonstrated in the lung extracts by complement fixation tests and titrations in mice and eggs.

3. Incubation at 37°C. was effective in bringing out agglutinins in high titer for chicken red cells in lung extracts, which originally failed to agglutinate chicken cells but agglutinated mammalian red cells. This method did not increase the titers of mammalian cell agglutinins.

4. The body temperature of the hamster was found to decrease within 1 to 4 days after inoculation of influenza virus. In the early passages the temperature returned to normal within 24 hours, but with the development of the pathogenic strain of virus the temperature remained at subnormal levels until death.

5. The Lee strain of influenza B virus produced pulmonary lesions in the hamster on the first passage and no increase in pathogenicity of the virus occurred during eleven serial passages. Virus was demonstrable in extracts of the lungs by all the methods used and no difference was observed in its capacity to agglutinate fowl and mammalian types of red cells.

The implications of these findings are considered briefly in the discussion.

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THE EFFECT OF CHICK EMBRYO EXTRACT ON THE GROWTH AND MORPHOLOGY OF TUBERCLE BACILLI

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PLATE 16

(Received for publication, June 22, 1948)

Minced embryonic tissue of chicken is known to facilitate growth of tubercle bacilli in synthetic media. Thus Friedmann obtained faster and more abundant growth of both human and bovine strains by adding chick embryo tissue to a basal medium (6). Soltys cultivated avian strains in Tyrode solution containing 2 per cent of chick embryo pulp. While the human and bovine strains studied in his experiments multiplied in the first subculture in this medium, they failed to grow in subsequent transplants (8).

The present publication describes the effect of embryo extracts on the rate of growth, morphology, and virulence of tubercle bacilli cultivated in the media recently developed in this laboratory.

EXPERIMENTAL

Methods.—The culture media used were those described by Dubos and Middlebrook (5). In addition to mineral salts, casein hydrolysate, and serum albumin, these media contain 0.005 per cent oleic acid (medium 1) or 0.05 per cent of the water-dispersible ester of oleic acid Tween 80 (medium 2). 11-day-old chick embryos were removed aseptically from the eggs, washed in distilled water, and minced in a Waring blender following addition of 1.5 cc. distilled water per embryo. The embryo pulp was centrifuged at 4°C. for 30 minutes at 3500 R.P.M. and the supernatant fluid was used,—chicken embryo extract (CEE). Extracts of muscle, lung, kidney, and spleen were prepared by a similar technique. The tissue extracts were added under aseptic conditions to the culture medium distributed in 5 cc. amounts in Pyrex glass tubes 25 mm. in diameter.

Most of the experiments were carried out with the human strains of tubercle bacilli H37Ra (avirulent) and H37Rv (virulent).¹ A few bovine and avian strains have also been tested and found to behave essentially like the human strains. The standard inoculum corresponded to a final 10^{-2} dilution of a fully grown culture in Tween-albumin medium (approximately 3×10^{-4} mg. dry weight bacilli per cc. of medium).

Effect of CEE on Growth Rate.—The data presented in Table I summarize the plan and results of a typical experiment. They show that addition of 0.5

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¹ These strains were originally obtained through the courtesy of Dr. W. Steenken of the Trudeau Sanatorium. They have been subcultured in our laboratory in the Tween-albumin medium.

to 1.0 per cent of CEE to the oleic acid-albumin medium renders growth more abundant and more rapidly detectable than in the control media not containing the extract. This was particularly striking when small inocula were

TABLE I

The Effect of Chick Embryo Extract on the Growth of Tubercle Bacilli in Oleic Acid-Albumin Medium

Final concentration of CEE	EXTRACT			EXTRACT		
	Time after inoculation, days			Time after inoculation, days		
	3	6	12	3	6	12
<i>Per cent</i>						
0	1 ⁺	4 ⁺	6 ⁺	2 ⁺	5 ⁺	7 ⁺
1.0	4	6	8	5	8	>8
0.5	4	6	7	5	7	8
0.25	2	5	7	3	6	7
0.125	1	5	6	2	6	7

* Inoculum: 5×10^{-4} mg. bacilli per cc. of medium.

† The amount of growth is recorded in terms of turbidity estimated visually according to an arbitrary scale from 0 (no growth) to 8 (growth corresponding to approximately 0.4 mg. dry weight of bacilli per cc. of medium).

TABLE II

Influence of Size of the Inoculum on the Growth-Promoting Effect of Chick Embryo Extract

Final concentration of CEE	EXTRACT Inoculum (mg. dry bacilli per cc. medium)								
	5×10^{-4}			3×10^{-4}			3×10^{-4}		
	Growth after varying incubation time, days								
	3	6	12	3	6	12	3	6	12
<i>per cent</i>									
0	2*	5*	7*	1*	2*	3*	0*	1*	1*
1.0	5	7	>8	4	6	7	1	5	5
0.5	5	7	8	2	5	6	0	2	5
0.25	2	6	7	1	5	4	0	2	2
0.125	2	5	6	1	5	5	0	1	1

* Symbols same as in Table I.

used (Table II). Although no quantitative study has been made of the rate of bacterial multiplication, it appears from microscopic observations and from the increase in turbidity of the culture that the most pronounced effect of CEE takes place during the logarithmic phase of growth and that the lag phase is not markedly shortened. The observation that the maximum yield of bacilli

lary growth is reached earlier in the presence of the extract is in agreement with findings reported by others (6, 8).

Effect of CEE on Bacterial Morphology.—Middlebrook *et al.* (7) observed a correlation between the virulence of different strains of mammalian tubercle bacilli and the pattern of growth of these strains—a pattern due to the formation of bacillary cords. The more virulent a strain, the more pronounced was its ability to grow in the form of bacillary cords (serpentine pattern of growth). It was noted during the present work that addition of CEE to oleic acid-albumin medium caused the bacilli to organize themselves in cords which were longer and more dense than those formed in the absence

TABLE III

Effect on Male Swiss Albino Mice of Intravenous Inoculation of Tubercle Bacilli, H37Rv (Virulent) and H37Ra (Avirulent), Grown in the Presence or Absence of Chick Embryo Extract
(0.03 mg. dry weight of bacilli was inoculated into each animal.)

Inoculum 0.03 mg. of	No. of mice	Death and survival											
H37Ra	12	S+	S+	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-
H37Ra (CEE)*	"	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S-
H37Rv	"	D43	D45	D47	D47	D50	D50	D51	D51	S+	S+	S+	S+
H37Rv (CEE)*	"	D21	D21	D21	D27	D39	D39	D40	D40	D43	D48	D48	S+

D, death; the number indicates the number of days after inoculation at which death occurred.

S, survival for a period of 57 days, at which time the animals were sacrificed.

The symbol + means that tuberculous lesions were present in the lungs.

The symbol - means that no pulmonary lesions could be detected macroscopically.

* These cultures were grown in the presence of chick embryo extract.

of the extract and within which the bacterial cells were arranged strictly in parallel (Figs. 3 and 4).

Whereas in the case of the virulent variant H37Rv the embryo extract caused only a quantitative difference in cord formation, differences of a qualitative order were observed with the avirulent variant. In ordinary media, the avirulent bacilli always grew in unoriented clumps, but they formed well defined cords in the presence of CEE (Figs. 1 and 2). However, the fact that the avirulent bacilli always remain less acid-fast than the virulent forms facilitates their identification even when they grow in the form of cords.

Experiment has shown that cord formation by H37Ra in the presence of CEE is not a transmissible modification but occurs only if enough extract to induce it is present in the medium during growth. Growth assumes again the usual unoriented pattern as soon as the strain is returned to ordinary culture media devoid of CEE.

These extracts also increase the tendency of virulent strains to exhibit the serpentine pattern of growth and confer this property on avirulent variants which normally grow unoriented in clumps.

Growth in the presence of chick embryo extract slightly increases the virulence of the virulent strains but does not confer virulence on the avirulent variants.

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EXPLANATION OF PLATE 16

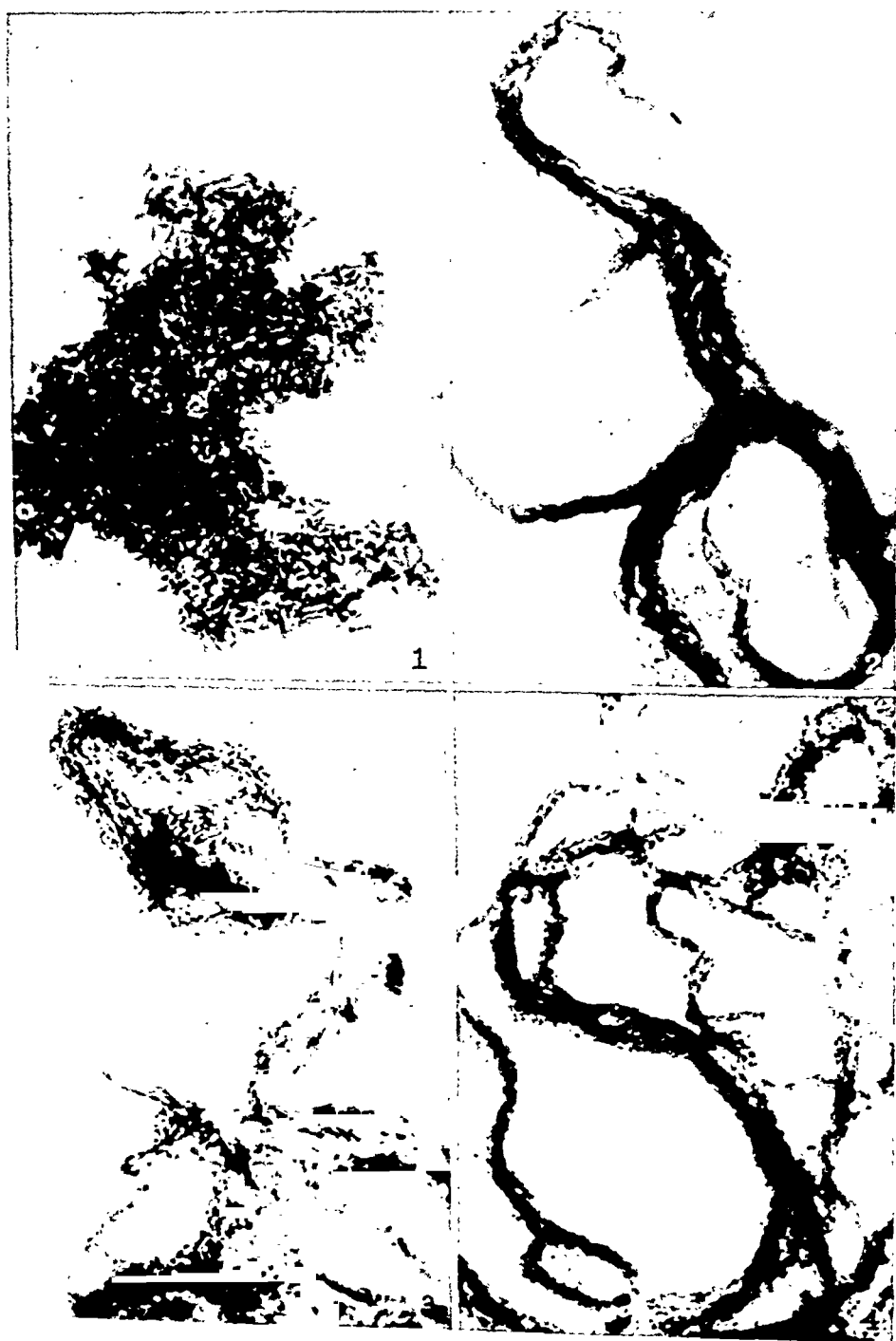
The photographs were made by Mr. Julian Carlisle.

FIG. 1. Ziehl-Neelsen-stained preparation of 8-day-old culture of avirulent tubercle bacilli (H37Ra), grown in oleic acid-albumin medium. The bacilli lie helter-skelter in clumps. $\times 1090$.

FIG. 2. Ziehl-Neelsen-stained preparation of avirulent tubercle bacilli (H37Ra), grown in oleic acid-albumin medium containing 0.5 per cent chick embryo extract. The bacilli are arranged in parallel and form cords. $\times 1090$.

FIG. 3. Ziehl-Neelsen-stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid-albumin medium. The bacilli form cords. $\times 1090$.

FIG. 4. Ziehl-Neelsen-stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid-albumin medium containing 0.5 per cent chick embryo extract. The cords are more dense than in Fig. 3 and the parallel arrangement of the bacilli is more pronounced. $\times 1090$.



(Bloch: Chick embryo extract and tubercle bacilli)

THE INFLUENCE OF RELATIVE HUMIDITY ON THE INFECTIVITY OF AIR-BORNE INFLUENZA A VIRUS (PR8 STRAIN)*

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(Received for publication, June 9, 1948)

INTRODUCTION

The observation that the degree of relative humidity has a significant influence on the survival of air-borne influenza A virus was first made by Loosli and associates in 1943 (1). They found that the duration of infectivity of this virus for white mice was greatest at a low humidity; *e.g.* 23 per cent, and much less at higher humidities. Further light on the influence of varying degrees of relative humidity on air-borne pathogens was provided by the work of Dunklin and Puck (2) who found that the death rate of pneumococci, hemolytic streptococci, and staphylococci atomized into the air of an experimental chamber, was exceedingly rapid at the middle humidities (45 to 55 per cent) and very much slower under conditions of both low and high relative humidities.¹

The general pattern of these observations was confirmed in the course of studies on the virucidal action of triethylene glycol for influenza A virus at various relative humidities. However, the early experiments revealed little evidence of the deleterious influence of intermediate humidities (40 to 60 per cent) on the infectivity of the air-borne virus (Loosli and Robertson (3)); whereas, more recent ones showed this effect in a regular and definite manner. It was apparent that some factor had been modified unwittingly and an attempt was made to determine the explanation of this difference.

The present investigation consists of a study of the effect of varying per cent relative humidities on the infectivity of air-borne influenza A virus as shown by the ability of this virus to produce death or lesions in white mice, under conditions in which the potency of the virus suspension, the dose of the virus, and the physical environment could be carefully controlled.

Materials and Methods

Mice.—Young white mice weighing 8 to 12 gm. were used in all experiments. These mice were of the Swiss strain and were obtained from Mr. Stanley C. Miller of Germantown, New York. They have been shown to be free from spontaneous pneumonitis.

* Aided by special grants from the Research Corporation and the United States Public Health Service.

¹ Dunklin and Puck have summarized earlier work on the effect of relative humidity on the survival of air-borne pathogens.

had been calibrated previously with a modified Cascade impactor by the technique described by Sonkin (7) and was found to deliver a cloud of 1.5μ mmd.² Since the conditions of atomization were kept constant throughout, the particle size distribution of the cloud issuing from the atomizer was essentially the same in all experiments. The 1 minute period of atomization regularly employed resulted in the dispersion of between 0.82 and 0.85 gm. of virus suspension as shown in Table II. By varying the duration of the spraying, the amount of suspension discharged into the chamber could be increased or decreased as desired. The atomizer was placed directly above the constant speed fan in the center of the chamber which pointed toward the ceiling and rotated at a speed of 500 R.P.M. This ensured adequate distribution of the virus throughout the air of the enclosed space. The mice were exposed to the virus for a period of 15 minutes at successive time intervals indicated in the protocol of the experiment shown in Table I.

TABLE I
*Protocol of Experiment Showing Schedule of Exposure of Mice to Minimal
Lethal Dosage of Air-Borne Influenza A Virus*

Cage	Time	Duration of exposure	Number of mice exposed	Fatalities	
				Number	Per cent
A	0-15	15	10	10	100
B	6-21	15	10	10	100
C	11-26	15	10	10	100
D	16-31	15	10	10	100
Total.....			40	40	100

Experiment 79:

Atomizer A-99 mmd. = 1.5 micra.

RH = 23 per cent.

Virus sprayed 1 minute at 400 mm. Hg.

Fan = 500 R.P.M.

Temperature = 74° F.

Weight of virus suspension delivered = 0.85 gm.

Autopsies were performed within 12 hours after death and the lesions found were recorded. All surviving mice were sacrificed 14 days after exposure and examined for pulmonary lesions. Deaths not clearly attributable to influenza virus were uncommon and in the particular series reported no deaths occurred in which the typical pulmonary lesions of influenza were not present.

RESULTS

Effect of Different Humidities on the Infectivity of Air-Borne Influenza A Virus

Preliminary experiments to determine the minimum lethal dosage of virus were carried out at low relative humidity, e.g. 20 to 25 per cent, since numerous previous observations indicated that influenza virus dispersed into dry air sur-

² Mass median diameter equals that diameter which represents the median value of the particle size distribution of the cloud, i.e., 50 per cent of the mass of the particles is below this particular diameter in size (Sonkin (7)).

vived longer than it did in a moist atmosphere. It was found that quantities of virus suspension as small as 0.80 to 0.85 gm. regularly produced death of all the exposed mice. A typical experiment is shown in Table I.

A series of experiments employing a dosage of 0.82 to 0.85 gm. of virus suspension was then carried out at different relative humidities. The results are summarized in Table II and Fig. 1 and show that an amount of atomized suspension which produced a 100 per cent mortality at 20 and 30 per cent relative humidity, caused death in only 22½ per cent of the mice exposed at humidities from 45 to 60 per cent. As the humidity was further raised this same amount of virus became increasingly infective until at a humidity of 80 per cent all the exposed mice died.

TABLE II
Infectivity of Air-Borne Influenza Virus at Various Relative Humidities

Relative humidity	Weight of virus suspension atomized	Number of mice in experiment	Fatalities		Survivors			
			Number	Per cent	Number	Per cent	Number having pulmonary lesions	Per cent having lesions
<i>per cent</i>	<i>gm.</i>							
23	0.845	40	40	100	—	—	—	—
30	0.828	40	40	100	—	—	—	—
40	0.827	40	38	95	2	5	2	100
45	0.834	40	9	22.5	31	77.5	12	38.7
50	0.833	40	9	22.5	31	77.5	8	25.8
55	0.840	40	9	22.5	31	77.5	9	29.0
60	0.835	40	9	22.5	31	77.5	20	64.5
65	0.839	40	17	42.5	23	57.5	21	91.3
70	0.831	40	30	75	10	25	10	100
80	0.850	40	40	100	—	—	—	—

The presence or absence of lesions among the surviving mice revealed more clearly the point of minimum infectivity which was found to be 50 per cent relative humidity (Table II and Fig. 1). Of the 40 mice exposed at this humidity, only 9 died, and, of the 31 survivors, only 8 exhibited lesions. At humidities just above and below this level the percentage of surviving mice with pulmonary lesions was greater although the mortality was the same as that occurring at 50 per cent relative humidity.

Effect of Increasing Dosage of Virus at a Relative Humidity of 50 Per Cent

A series of experiments in which the virus suspension was atomized in increasing amounts from 0.85 gm. to 2.91 gm. was performed at a humidity of 50 per cent in an attempt to secure a 100 per cent mortality. The results, shown in Table III, reveal that when 0.85 gm. of the suspension was atomized

the mortality rate was 22.5 per cent and when the amount was increased to 2.91 gm. the mortality rate rose to only 52.5 per cent. In these experiments the increase in amount of virus dispersed was secured by lengthening the duration of atomization. However, the loss of infectivity of the air-borne virus at this humidity was so rapid that it became impractical to atomize for a period of time

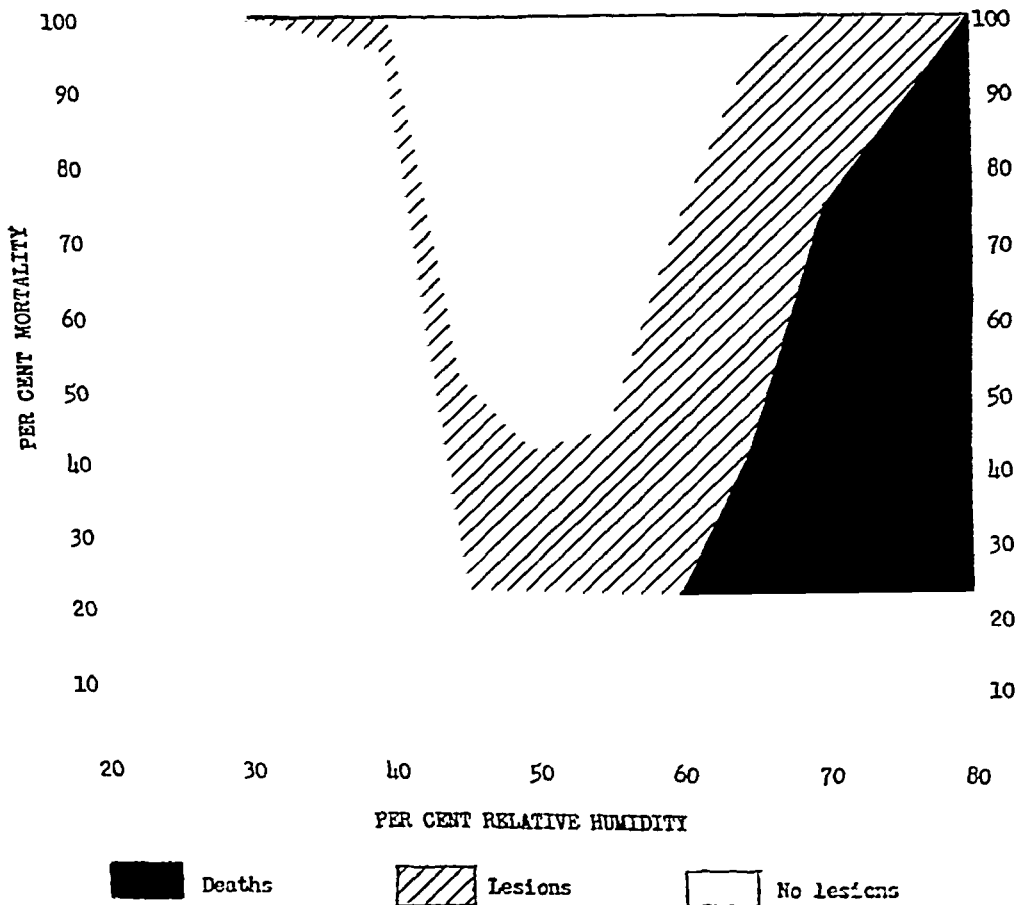


FIG. 1. Effect of relative humidity on the infectivity of air-borne influenza A virus.

greater than $4\frac{1}{2}$ minutes because under those conditions the loss of activity approximates the rate at which the virus could be atomized into the air.

Absence of Demonstrable Influence of Humidity on Dialyzed Virus Suspension

Once the pattern of the influence of relative humidity was established it became of interest to attempt to modify or eliminate it. Direct analogy from

that survival was prolonged at humidities below 40 per cent or above 70 per cent. Broth suspensions of influenza A virus showed the same general response when atomized into the experimental chamber at differing relative humidities. The infectivity of the atomized virus suspension decreased markedly as the humidity was raised from 30 to 50 per cent and then gradually increased as the humidity was further elevated to 70 per cent and at 80 per cent again was maximum.

Even more interesting was the observation that diminishing the salt concentration of the virus-lung suspension by dialysis, eliminated the deleterious influence of humidity in the range of 50 per cent on the infectivity of the atomized virus.

As was mentioned previously, early experiments showed little evidence of the deleterious influence of the intermediate humidities (40 to 60 per cent) on the infectivity of air-borne influenza A virus whereas the data cited in this paper demonstrated this effect in a pronounced manner. The finding that the dialyzed virus suspension did not show the influence of humidity led to a possible explanation of this discrepancy. Examination of the experimental protocols revealed that the virus suspensions used in the earlier experiments by Loosli and Robertson (3) were diluted with heart infusion broth prepared in the laboratory and containing no added sodium chloride. The suspensions used in the present series were diluted with Difco heart infusion broth which contained a minimum of 5 gm. of sodium chloride per liter. This observation would indicate that the concentration of sodium chloride in the virus suspension may be a critical factor in determining the infectivity of influenza virus by the air-borne route. The elucidation of the precise mechanism of this phenomenon must await further study.

The epidemiologic implications of these observations on influenza A virus, as well as those of Dunklin and Puck on bacteria, may well be important. It seems likely that, in most environments, greater numbers of the infective agents dispersed from the respiratory tract are destroyed than survive and many factors undoubtedly play a rôle in this process. The results of this study suggest that the relative humidity may constitute one of the important factors in determining the survival of respiratory pathogens issuing from an infected host.

SUMMARY

White mice were exposed to atmospheres containing known amounts of atomized influenza A virus (PR8 strain) of constant potency under conditions of varying humidity. It was found that an amount of atomized virus suspension which produced a 100 per cent mortality rate in animals exposed at 30 and 80 per cent relative humidity, respectively, resulted in the death of only 22.5 per cent of mice at a humidity of 50 per cent. The humidities between these values gave intermediate results.

The infectivity of the air-borne virus decreased so rapidly at a humidity of 50 per cent that it was impossible to secure a 100 per cent mortality rate in the exposed mice even by greatly increasing the dose of virus atomized.

The use of a dialyzed virus suspension at a humidity of 50 per cent resulted in the death of all exposed mice. This suggested that the deleterious influence of humidity was related to the presence of sodium chloride in the atomized suspension.

These findings with influenza virus closely resembled those obtained by Dunklin and Puck with pneumococci, streptococci, and staphylococci which would suggest that the factor responsible for the lethal effect of humidity is common to moist particles containing either the above-mentioned bacteria or influenza A virus.

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THE HUMAN ANTIBODY RESPONSE TO SIMULTANEOUS INJECTION OF SIX SPECIFIC POLYSACCHARIDES OF PNEUMOCOCCUS*

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Previous quantitative studies of the antibody content of the sera of human beings injected with immunizing (1) doses of the specific polysaccharides of pneumococcus have dealt with analyses of sera after injection of two or three (reference 2) and four (reference 3) specific polysaccharides at one time. Since it was feasible for practical purposes to combine into a single solution as many as six polysaccharides of the pneumococcal types most commonly responsible for pneumococcal pneumonias in man, exact knowledge of the antibody response to the injection of so many chemically similar antigens appeared desirable. Data obtained with two different combinations of six polysaccharides are given in the present report.

EXPERIMENTAL

Medical student volunteers, after a preliminary bleeding (subscript 0), were injected subcutaneously with 1 ml. of a solution containing about 0.07 mg. each of six type-specific polysaccharides of pneumococcus. Subjects 201 to 206 received the specific polysaccharides of Types I, II, III, V, VII, VIII pneumococcus, these being the types most common in pneumococcal pneumonia of adults, while Nos. 207 to 212 received Types I, IV, VI, XIV, XVIII, XIX, the types most common in the pneumococcal pneumonias of children. Both antigen solutions were prepared by E. R. Squibb and Sons. Subject J, a woman 63 years of age, received the first solution. Nine months after its injection, sample J₁ was taken, when the antibodies formed were probably no longer at peak levels (2). From the other subjects a bleeding (subscript 1) was taken 6 weeks to 2 months after the injections. Analyses on sera 202₁ and 210₁ could not be completed owing to accidental contamination, and additional samples 202₂ and 210₂ were procured 9.5 and 5.5 months after injection, respectively.

Analyses were carried out according to the method mentioned in reference 4 and described in detail in reference 2. Precautions against bacterial contamination were taken owing to the large number of successive analyses required and the long standing necessary for each analysis. Superscripts after values for antibody nitrogen indicate an unusually large number of absorptions for complete removal of the antibody in question.

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DISCUSSION

The analytical values for the type-specific antibody content of the sera of human beings injected subcutaneously with a mixture of six type-specific polysaccharides of pneumococcus resemble those obtained in earlier quantitative micro-

TABLE I
Micrograms Antibody Nitrogen Per 4 Ml. Pre- and Post-Immunization Sera of Subjects Injected with Specific Polysaccharides of Pneumococcus Types I, II, III, V, VII, and VIII, 0.05 to 0.1 Mg. Each

Antibody to	C III	C VI	S I	S II	S III	S V	S VII	S VIII	S XIV
201 ₀	19	17	0	5	10*, 15	0	0	4, 3	3
201 ₁		53	31	64	29	21	49 [†]	7	
202 ₀	27	21	0	3	4*, 4	0	0	0, 0	0
202 ₁		35			(14)†		15 [†]	6	
202 ₂		40	3, 3	21	1*, 11	0, 0	0	4, 1	
203 ₀	39	30	0	1	4*, 3	0	1	0, 0	11
203 ₁		52	33	33	21	107	36 [†]	39 [†]	
204 ₀	20	14	0	0	0*, 0	0	0	0, 0	0
204 ₁		16	3	6	2	4	11 [†]	18	
205 ₀	46		0	0	1*	0	3	6	
205 ₁		62	15	21	11	1	30 [†]	8	
206 ₀	32		0	0	0*	0	2	3	
206 ₁		28	8	18	5	0	7 [†]	1	
J ₀	23		0	0	4*	0	0	0	
J ₁	16	15	13	10, 6	12*, 16	0, 8	<31	17	

C III = C-substance derived from Type III pneumococcus. C VI, from Type VI; S I, S II, etc., refer to type-specific polysaccharides.

* After removal of anti-C with C-substance derived from Type III and possibly containing traces of S III.

† Last analysis before contamination noted.

analytical studies of the sera of subjects injected with two or three or four of the antigens at a time (2, 3). The total antibody response and the production of antibody to each antigen injected appeared to be as satisfactory as in the subjects treated with smaller numbers of polysaccharides. Variability of response and unequal reactivity of an individual to all of the type-specific polysaccharides in the mixture were encountered as frequently as in the previous series.

In marked contrast to the behavior of the groups previously studied, roughly

one-half of the subjects showed increases in the anti-C content of their sera after the injections. Since almost all of the specific polysaccharides contain C-substance as impurity, it is possible that the intensity of the antigenic stimulus for production of additional amounts of anti-C increases with the number of polysaccharides used, and, hence, the probably greater quantity of C-substance injected. Whether or not all of this or only a portion is antigenic in man is not known.

TABLE II

Micrograms Antibody Nitrogen Per 4 ml. Sera of Subjects Injected with Specific Polysaccharides of Pneumococcus Types I, IV, VI, XIV, XVIII, and XIX, 0.05 to 0.1 Mg. Each

Antibody to.....	C	S I	S III*	S IV	S VI	S XIV	S XVIII	S XIX
207 ₀	27	0	0	0	0	0	3	0
207 ₁	26	54		9	2	47	9	6
208 ₀	6	0	0	0	0	0	5	0
208 ₁	55 ^a	24		16 ^a	4	0	52	0
209 ₀	31	0	0	2	1	4	5	1
209 ₁	89	24		48 ^a	18	56	11	15 ^a
210 ₀	87	0	11	0	0	7	16	0
210 ₁	158	50		23				
210 ₂	199	26		21	58 ^a	23	35	44 ^a
211 ₀	29	0	1	0	0	4	0	0
211 ₁	32	5		5	1	9	5	0
212 ₀	56	4	3	1	1	6	2	0
212 ₁	57	19		5	8	70	9	14

* S III was not in the mixture injected. Additional data on normals were sought.

Attention is also directed to the relatively large proportion of the subjects whose pre-immunization sera contained measurable quantities of antibodies which precipitate with the specific polysaccharides of Types XIV and XVIII pneumococcus.

SUMMARY

1. The antibody response in human beings after the injection of six type-specific polysaccharides of pneumococcus appears to be roughly of the same magnitude for each type as after the injection of fewer antigens.

2. A larger proportion of increases in anti-C was noted in the present series.

3. Many of the pre-immunization sera tested contained antibodies reactive with the specific polysaccharides of Types XIV and XVIII pneumococcus.

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PRODUCTION OF ATHEROMATOSIS IN THE AORTA OF THE BIRD BY THE ADMINISTRATION OF DIETHYLSTILBESTROL*†

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PLATES 17 TO 21

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Although the concentration of lipids in the blood and liver of the male and immature female bird does not differ greatly from the range found in other species such as man and dog, avian lipid metabolism differs from mammalian in being under the control of ovarian hormones (1, 2). Thus, in the bird actively engaged in egg-laying there occurs a rise in the levels of fat, phospholipid, and cholesterol of the blood; in this state total lipids of the blood may rise as high as 4000 mg. per 100 cc., as compared with values of 500 mg. in the non-laying state. This rise in the various lipid constituents of the blood could be reproduced in the immature female or male bird by the injection of such estrogenic compounds as estrone, estradiol, and stilbestrol. In stilbestrol-treated birds, values for plasma lipids as high as 10,000 mg. per cent have been observed (3). The response of the plasma lipids to this estrogen does not depend upon the fat content of the diet; it was shown to occur in starved birds as well as in those fed a fat-free diet (3).

This distinctive feature of the lipid metabolism of the bird made possible the development of a new method for the study of experimental atherosclerosis. It is shown here that atheromatosis can be induced in the bird by the administration of diethylstilbestrol, a procedure that results in a sustained hyperlipemia. An extensive study of this estrogen-induced lesion is presented here. In addition, a comparison has been made between it and the atheromatosis induced in the bird by the feeding of cholesterol.

Materials and Methods

Thirty-six single comb White Leghorn cockerels reared on the low fat Poultry Division stock, diet (1) were divided at 3.3 months of age into 3 groups. Pellets of fused diethylstilbestrol, each weighing approximately 25 mg., were implanted subcutaneously in the birds of one group; each bird received a single pellet at the start of the experiment and another 30, 70, and 95 days later. The birds of a second group were fed cholesterol at a level of 2 per cent in the diet.

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An amount of cottonseed oil sufficient to dissolve the cholesterol was used and mixed with the Poultry Division stock diet. The third group of birds was maintained on the stock diet as controls.

The birds were weighed at intervals, and from time to time blood samples were removed from the alar vein. Blood was drawn into heparinized syringes, centrifuged, and the plasma separated. The concentration of cholesterol, phospholipids, and total fatty acids in this plasma was determined by a procedure described elsewhere (4). Free cholesterol was determined in an acetone solution from which phospholipids had been precipitated.

The birds were sacrificed by exsanguination. The aorta and major aortic branches were removed together. After fixation in 10 per cent solution of formaldehyde U.S.P., the vascular apparatus was opened longitudinally and its gross appearance noted. Blocks of tissue were removed from each aorta at the following levels: apex of arch, interadrenal region, and about 1 cm. above the aortic bifurcation. Contiguous frozen sections cut from each block were stained with Sudan IV and hematoxylin (5) and Nile blue (6). An unstained frozen section was used for examination with polarized light. These blocks were then embedded in paraffin, and contiguous sections treated with hematoxylin and eosin stain, Laidlaw's connective tissue stain, and a combined Verhoeff-van Gieson stain (6).

Arteriosclerosis in Untreated Male Birds: the Control Group

Spontaneous arteriosclerosis has repeatedly been observed in the bird (7-12). The gross and microscopic appearance of the aortas of the 12 control male birds studied here is summarized in Table I.

Thoracic Aorta.—

Although grossly visible lesions occurred rarely in the thoracic aortas of our male birds (Table I), a minimal amount of lipid material was, however, visible microscopically in the intima of this portion of the aorta in 4 birds (Fig. 1). This minimal lesion, which is shown in Fig. 1, was unaccompanied by fibrous thickening. It consisted of deposits of fine lipid droplets in the connective tissue cells of the intima and adjacent portions of the media. As judged by examination with polarized light, cholesterol was absent in this lesion.

The thoracic aortas of 8 of the birds were free of stainable lipid material (Table I). An example is shown in Fig. 2.

Abdominal Aorta.—

Nine birds of this group (Table I) showed either gross and/or microscopic evidence of intimal thickening in the lower part of the abdominal aorta in the area just above the bifurcation. The lesion consisted of longitudinal ridge-like plaques 1 to 2 mm. wide and 7 to 15 mm. long. The gray or white color of this lesion indicates its fibrous nature. The earliest lesion was usually found on the anterior wall of the artery, whereas the more advanced plaques were found on both anterior and posterior walls.

Early and late stages of this type of spontaneous arterial disease are shown in Figs. 3 to 8. The larger or more advanced lesion (Figs. 5 to 8) consisted of

dense, wavy, hyalinized connective tissue fibers running parallel to the endothelium covering the plaque (Fig. 6). The fibroblasts between the collagenous fibers were enlarged (Fig. 6) and contained numerous small lipid droplets, particularly in the deep portions of the plaques (Fig. 7). Fig. 8, which is a view

TABLE I

The Spontaneous Lesion Found in the Aorta of Control Male Birds

(All birds were 3.3 months old at start of experiment)

Bird	Age when sacrificed	Thoracic aorta				Lower abdominal aorta					
		Number of grossly visible plaques	Microscopic examination			Grossly visible plaques			Microscopic examination		
			Intimal thickening	Lipids*	Cholesterol†	Number	Size	Color	Intimal thickening	Lipids*	Cholesterol†
	mos.						mm.				
15-02	11	0	0	0	0	0			1+	1+	0
15-27	8	0	0	1+	0	0			0	0	0
15-28	7	3§	0	2+	0	0			0	0	0
15-55	11	0	0	0	0	0			1+	1+	0
15-79	11	0	0	0	0	1	1 × 5	White	3+	2+	1+
15-81	8	0	0	1+	0	0			2+	1+	1+
15-82	11	0	0	0	0	0			2+	2+	0
15-90	11	0	0	0	0	2	2 × 10	Gray	4+	2+	0
							1 × 10	White			
15-91	11	0	0	0	0	2	2 × 15	Yellow	4+	2+	1+
							1 × 12	Gray			
15-94	11	0	0	1+	0	1	1 × 7	Grayish white	4+	2+	1+
15-99	11	0	0	0	0	0			2+	2+	0
15-88	11	0	0	0	0	0			0	0	0

* As judged by Sudan IV and Nile blue stains.

† Estimated by polarized light and Nile blue stain.

§ Each plaque measured 0.5 × 0.5 mm. and was yellow in color.

Histologic grading of lipid or cholesterol in artery:

1+ = minimal amounts in intima only.

2+ = present in media as well as intima but not extensively.

3+ = moderate amounts in intima and media.

4+ = abundant in intima and media.

of a lesion with polarized light, demonstrates that some of this lipid is cholesterol.

Although the above description refers only to the abdominal lesion in young male birds, this same lesion has also been observed in the female bird (12).

The difference between the spontaneous lesions found in the thoracic and abdominal portions of the aorta is quite striking. Whereas that in the thoracic aorta consisted entirely of lipid material, the abdominal one, on the other hand, contained small amounts of lipids and appeared primarily fibrous in nature.

TABLE II
Lesions Found in the Aortas of Stilbestrol-Treated Male Birds
 (All birds were 3.3 months old at start of experiment)

Bird	Age when sacrificed	Thoracic aorta						Lower abdominal aorta					
		Grossly visible plaques			Microscopic examination			Grossly visible plaques			Microscopic examination		
		Number	Size	Color	Intimal thickening	Lipids	Cholesterol	Number	Size	Color	Intimal thickening	Lipids	Cholesterol
	mos.		mm.						mm.				
15-08	11	2	15 × 1	Yellow	3+	3+	2+	0			0	0	0
			0.5 × 0.5	"	1+								
15-10	11	1	2 × 2	"	3+	3+	1+	0			1+	1+	0
15-24	6	0			0	1+	0	Not examined					
15-26	11	4	12 × 2	"	3+	2+	2+	1	12 × 1	Yellow	3+	3+	2+
			4 × 2	"									
			1 × 1	"									
			1 × 1	"									
15-30	10	5	All 2 × 2	"	2+	2+	1+	Not examined					
15-35	8*	3	0.5 × 0.5	Orange	3+	3+	0	Not examined					
15-36	9	0			2+	2+	1+	1	1 × 5	"	2+	3+	2+
15-45	11	1	1 × 1	Yellow	2+	2+	2+	0			1+	2+	0
15-48	11	Numerous†	Coalescing	"	4+	4+	4+	0			1+	1+	1+
15-58	6	0			0	1+	0	0			1+	1+	0
15-65	11	0			0	2+	2+	2	0.5 × 0.5	"	4+	4+	2+
									1 × 7	"			
15-85	8	0			0	3+	3+	Not examined					

* One pale orange plaque 1 × 1 mm. was found in right brachiocephalic artery.

† The entire thoracic aorta was involved. Numerous longitudinal lesions were also found in both brachiocephalic arteries.

Arteriosclerosis in Stilbestrol-Treated Male Birds

Thoracic Aorta.—

A considerable deposition of lipids was observed in the thoracic aortas of a 12 birds of this group (Table II). As noted above, this condition was found in 4 of the 12 control birds, but only to a minor degree.

Gross Appearance of the Thoracic Lesion.—Grossly visible lesions were found in 7 of the 12 injected birds. In 4 of them (15-08, 15-10, 15-26, and 15-45) one or more ovoid, slightly elevated intimal plaques were found on the convex side of the aortic arch about 1 cm. from the aortic valve. These lesions were lemon yellow in color, and measured from 1 to 2 mm. in width and as much as 15 mm. in length. When more numerous, they had a tendency to coalesce. This was particularly evident in bird 15-48, in which many coalescing elevated yellow intimal lesions were present in the brachiocephalic arteries and throughout the aorta. In

bird 15-35, which was sacrificed 8 months after the injection of stilbestrol was begun, the most prominent lesion was a single plaque in the left brachiocephalic artery. In still another bird (15-30) small areas of the intima of the thoracic aorta were found to have a lemon yellow color, but these areas were neither elevated nor plaque-like.

Microscopic Appearance of the Thoracic Lesion.—The significant microscopic finding was a lipid infiltration in the connective tissue cells of the intima and adjacent media (Figs. 10, 12, 16, 17). The lipids were present as small droplets within the cytoplasm (Figs. 12, 17), and the bulk of it, when stained with Nile blue, was light pink in color. Moderately abundant, refractile, rod-shaped, or rectangular crystals were visible with polarized light in these layers (Figs. 13, 18). These lipid-infiltrated cells had a vacuolated cytoplasm (Fig. 15) when stained with hematoxylin and eosin. The lining endothelial cells were often swollen and vacuolated, and lipid globules could be demonstrated with specific stains.

Microscopic examination revealed the presence of thick intimal plaques in the aortas of 8 of the birds (Figs. 9, 14). The thickened intima consisted of large vesicular fibroblastic cells, supported by reticulum and collagenous fibers running perpendicular to the endothelial surface (Fig. 11). The more deeply situated fibers were collagenous and condensed. In addition, these intimal plaques contained distinct foam cells of the macrophage type. The newly formed intimal connective tissue cells likewise were filled with lipid material. There was no mitotic evidence of fibrous cellular proliferation. In 8 of the 12 birds small to moderate numbers of refractile rod-shaped or rectangular crystals were seen with polarized light (Fig. 13). In a single stilbestrol-treated bird (15-48) greater amounts of cholesterol were discernible with polarized light than in any of the cholesterol-fed birds (Fig. 18). No alteration of the elastic tissue in the thoracic aorta was found in any of the stilbestrol-treated birds.

Upper Portion of Abdominal Aorta.—

Gross Appearance.—In 5 birds, grossly visible lesions were found in the upper portion of the abdominal aorta at the level of the adrenal glands. In one bird (15-48) the lesion resembled that seen in the aortic arch. In the other 4, the lesions were small and few in number. They consisted of slightly elevated, rounded pale yellow or orange plaques which measured less than 1 mm. in diameter.

Microscopic Appearance.—In 8 birds, intimal thickening was demonstrated. In 5 birds this was minimal and consisted of a thin layer of vacuolated fibroblastic cells supported by coarse reticular fibers lying between the endothelium and the media. These cells, as well as some of the adjacent medial cells, contained lipid droplets. In only one bird (15-26), however, was it possible to demonstrate with polarized light a few spindle-shaped refractile crystals. In 3 birds, the intimal thickening was more prominent, usually leading to formation of flattened plaques on the anterior or posterior walls. In general, they consisted of fibroblastic cells supported by collagenous and reticulum fibers which tended to run parallel to the endothelial surface. The fibroblastic nuclei were flattened and lay in clear fusiform spaces, which were enclosed by connective tissue fibers and contained lipid droplets. In the larger plaques the deeper portions were more cellular, and these cells contained more lipid substance. In addition, the supporting reticulum and collagenous fibers in the larger plaques tended to run perpendicular to their surfaces. In the largest intimal plaques found in 3 birds, spindle-shaped and rectangular refractile crystals were abundant. In birds in which intimal lipid deposition was abundant, moderate amounts of lipid substances were also present in the media. Most of this was intracellular, but some apparently lay in extracellular situations. Occasionally groups of lipid-containing foam cells of fibroblastic type were present in the medial layer beneath the intimal plaques. There was no alteration of the elastic tissue, and the endothelial cells were not remarkable.

Lower Portion of the Abdominal Aorta.—

Gross Appearance.—The lower portion of the aorta above the bifurcation was examined in only 8 birds (Table II). Three showed a ridge-like plaque on the anterior wall. The lesions of all 3 were distinctly lemon-yellow in color. These plaques measured approximately 1 mm. in width and between 7 and 12 mm. in length.

Microscopic Appearance.—Microscopically demonstrable intimal lesions were found in 7 birds (Table II; Figs. 19 to 23). Such lesions were composed of fibroblastic cells with compressed nuclei lying in clear spaces between wavy compact collagenous fibers. The latter tended to run parallel to the endothelial surface. Within the connective tissue cells were numerous lipid droplets. Only minimal amounts of refractile crystalline material were seen with polarized light in these microscopic lesions. In the birds not showing intimal lesions the media contained moderately abundant intracellular lipid globules.

The grossly visible ridge-like plaques that were found on the anterior arterial wall of 3 birds were almost large enough to obliterate the lumen (Fig. 19). In these birds the intima of the posterior and lateral walls was only mildly thickened and consisted of vesicular fibroblastic cells containing lipid and supported by circumferentially arranged collagenous fibers.

The large anterior plaques referred to above resembled the spontaneous lesion but showed, in addition, the following changes. The deeper portions were relatively acellular and consisted of dense hyalinized collagenous fibers enclosing fusiform spaces (Fig. 20). The latter contained vesicular fibroblastic cells. The long axis of the cells and fibers ran transversely. The fibers in the central portions of these plaques were swollen and homogeneous. Pools of vacuolated, protein-containing fluid lay between many fibers. Near the periphery were small calcific granules. The portion of the plaque adjacent to the endothelium was more cellular and consisted of vesicular foam cells of the fibroblastic type (Fig. 21). These were supported by delicate reticulum fibers tending to run perpendicular to the endothelium. The latter was intact, and the cells were not altered. Sudan IV stain revealed abundant bright orange lipid globules in the plaque, most numerous in the deeper and central portions (Fig. 22). In the latter situation, large pools of extracellular lipid material were evident. Considerable amounts of lipid substance were also present in the medial layer. Polarized light revealed moderate amounts of refractile rod-shaped or spindle-shaped or flat rectangular crystalline material in the plaque as well as in the thickened intima (Fig. 23). Beneath these large plaques the internal elastic membrane was thin.

*Arteriosclerosis in Cholesterol-Fed Male Birds**Thoracic Aorta.*—

Gross Appearance.—In all 12 birds of this group numerous intimal plaques were found in all portions of the aorta (Table III). In the thoracic portion of all but one (bird 15-89) these appeared as small, elevated, longitudinal streaks or pinpoint deposits. These lesions were most numerous on the convex surface of the intima, where they tended to coalesce. In most of the birds, the lesions were pale yellowish tan in color, although in two of them the plaques were yellowish white or yellowish orange in color. Similar intimal lesions were often visible in the cusps of the aortic valves, in sinuses of Valsalva, and in the brachiocephalic, carotid, subclavian, and femoral arteries.

Microscopic Appearance.—All birds showed microscopic atheromatous lesions in the thoracic aorta (Fig. 24). There was a generalized intimal thickening with an increase in the number of intimal cells, most marked in those zones where grossly visible intimal plaques were present. Between the plaques were deep indentations. The endothelium was usually intact, but in one instance had ulcerated and a small mural thrombus had formed. The endothelial cells were as

a rule enlarged and often had vesicular cytoplasm in which lipid droplets could be demonstrated. The thickened intima consisted mainly of large cells of the fibroblastic type with oval, pale, vesicular nuclei and rather abundant clear, or occasionally granular, cytoplasm. Connective tissue fibers lay between these cells and tended to run perpendicular to the endothelial surface (Fig. 25). Near the latter, they had a fine texture and corresponded to reticulum. In the deeper intimal layer they were coarse and collagenous. In one bird there was a zone of fibrocartilagenous and hyalin-cartilagenous metaplasia of a portion of the thickened intimal con-

TABLE III

Lesions Found in Aortas of Cholesterol-Fed Male Birds

(All birds were 3.3 months old at start of experiment)

Bird	Age when sacrificed	Thoracic aorta						Lower abdominal aorta					
		Grossly visible plaques			Microscopic examination			Grossly visible plaques			Microscopic examination		
		Number	Size	Color	Intimal thickening	Lipids	Cholesterol	Number	Size	Color	Intimal thickening	Lipids	Cholesterol
	<i>mos.</i>								<i>mm.</i>				
15-07	11	Numerous	Coalescing	Tan	3+	3+	2+	One*	1 × 6	Yellow-tan	4+	4+	4+
15-18	10	"	"	"	3+	2+	3+	"	1 × 4‡	Yellow-tan	2+	2+	3+
15-19	8	"	"	"	2+	2+	1+	2	Each 1 × 4	Tan	2+	3+	3+
15-38	8	"	"	"	1+	2+	2+	2	Each 1 × 5	"	2+	3+	1+
15-50	11	"	"	Orange	3+	4+	2+	Numerous	1 × 6	Yellow-white	4+	4+	4+
15-60	9	"	"	Tan	3+	3+	3+	One*	2 × 7	Tan	3+	3+	4+
15-67	8	"	"	"	3+	3+	3+	"	1 × 5	Orange	2+	3+	3+
15-72	10	"	"	"	3+	3+	3+	"	1 × 8	White	4+	4+	4+
15-84	11	"	"	"	3+	3+	2+	"	3 × 15	Yellow	4+	4+	4+
15-86	11	"	"	"	3+	3+	3+	"	1 × 10	Tan	4+	4+	4+
15-89	11	0			1+	1+	1+	"	2 × 7	Grey and yellow	4+	2+	2+
16-00	8	Numerous	Coalescing	Tan	3+	4+	3+	"	2 × 6	Tan	4+	4+	2+

* Both thoracic and abdominal portions contained small coalescing tan intimal plaques in addition to the single ridge.

‡ This single plaque extended from the level of the adrenal glands to the bifurcation.

nective tissue. The thickened intima contained no elastic tissue. A few of the intimal cells were macrophages which contained lipid material; these cells had small compact rounded nuclei. A few lymphocytes were present. When stained with Sudan IV, all the intimal cells and many of the medial connective tissue cells were shown to be richly supplied with bright orange lipid droplets which filled the cytoplasm (Figs. 26, 27). Smaller amounts of lipid material lay in extracellular situations. The amount of fatty material decreased toward the outer portion of the media, but was present as far as the adventitia. Large masses of refractile crystals were visible with polarized light in the intima and adjacent media (Fig. 28); these were either spindle-shaped or flat, notched, and rectangular. There was no alteration of the medial elastic tissue of the thoracic aorta.

Upper Portion of the Abdominal Aorta.—

Gross Appearance.—This portion of the aorta contained small tan plaques similar to those found in the thoracic aorta. They were less numerous and smaller than those in the thoracic aorta and hence showed less tendency to coalesce.

Microscopic Appearance: The Intima.—Sections from the aorta at the level of the adrenal glands showed a thickened intima composed mainly of enlarged lipid-containing fibroblasts which were supported by a delicate collagenous and reticulum network. These fibers ran perpendicular to the endothelial surface and were usually more abundant and compact at the periphery of the intimal plaques. Occasional lipid-filled macrophages were noted. Several aortic branches were occluded by a similar intimal fibrous thickening. A few small lipid droplets were present in and between the muscle cells of the adjacent media. There were numerous refractile rods in the thickened intima when viewed with polarized light.

Beneath the widened intima, the internal elastic membrane was narrowed or absent in some areas.

The Media.—The media was narrowed and small intimal calcific masses were present in those areas where the intimal thickening was particularly marked.

The Plaque.—In several birds the anterior wall at this aortic level was the site of an intimal plaque which histologically appeared similar to the spontaneous abdominal lesions described for the control group. These were composed of moderately dense connective tissue fibers, lying parallel to the circumference of the vessel and enclosing compressed fibrocytic nuclei. The latter were surrounded by clear fusiform spaces filled with lipid droplets which stained bright orange with the Sudan IV and blue to pink with the Nile blue. A few lipid droplets lay also in the muscular cells of the adjacent media. A moderate amount of refractile crystalline material was present in these intimal plaques. In several instances, the inner portion of the plaque adjacent to the lumen was composed of large, lipid-filled, fibroblastic foam cells supported by a delicate collagenous and reticulum stroma. These cells were abundantly supplied with lipids including refractile material, and were identical with the intimal cells of the thoracic aorta. In a few birds, the intimal alteration at the level of the adrenal glands approached in severity that seen in the lower aorta above the bifurcation.

Lower Portion of the Abdominal Aorta.—

Gross Appearance.—As already noted, the intimal plaques of this area were less numerous, smaller, and showed less tendency to coalesce than those described above. In the abdominal aorta of all birds in this group, there were large longitudinal, ridge-like plaques in the interrenal region. These plaques most frequently lay on the anterior wall and measured 1 to 2 mm. in width and 4 to 8 mm. in length. Infrequently, the posterior wall was similarly involved, though here the plaques were multiple, tended to fuse, and were tan-colored.

The anteriorly situated plaques were usually yellowish, yellowish orange, or white in color; a few, however, were tan-colored.

As a result of such extensive plaque formation, the lower abdominal aorta was usually quite rigid.

Microscopic Appearance.—Striking changes were observed just above the aortic bifurcation. The thickening of the intima on the anterior wall was usually extensive, forming a large plaque which almost occluded the lumen of the vessel (Figs. 29 to 32). The posterior intima was also thickened (Fig. 29) and consisted of fibroblastic cells with abundant clear cytoplasm. These cells were supported by moderately dense, edematous, collagenous and reticulum fibers. The long axis of the nuclei and the intercellular fibers tended to run perpendicular to the endothelial lining of the vessel. This thickened intimal tissue extended laterally on both sides, reaching the lateral portions of the large plaque lying in the anterior wall.

In the central portions of the large anterior plaque, the connective tissue adjacent to both endothelium and media consisted of a thin compact layer of flattened fibroblasts and collagenous fibers running parallel to the circumference of the vessel. This resembled the connective tissue arrangement of the spontaneous abdominal lesions. The endothelium was intact throughout, though the lumen was narrowed, slit-like, and crescentic.

Beneath the large anterior plaque the media was atrophic and mildly fibrotic (Figs. 29, 32). In some instances the media was absent, and masses of foam cells were found extending into the adventitia. The internal elastic membrane was absent, and the external one was thin and fragmented (Figs. 29, 32).

The bulk of the plaque was composed of pale, eosinophilic, fibrillary, almost mucoid, connective tissue containing very few flattened fibroblastic cells. Many of these were vacuolated (Fig. 33). Some of these cells were degenerating and enclosed numerous large clear fusiform spaces often arranged in a transverse herringbone manner (Figs. 30, 35). Small calcific granular masses were present at the periphery of the plaques (Figs. 32, 35). Occasionally the central portion of the plaque contained condensed fibrillary fibrin. Beneath the condensed connective tissue adjacent to the endothelium were moderate numbers of macrophages with abundant vesicular cytoplasm (Fig. 34). These were not enveloped by reticulum fibers. In the degenerated central portion of the large plaques, the reticulum and collagenous fibers were frayed, split, and often fragmented. Lipid material (Fig. 30) was abundantly present in the connective tissue cells of the thickened intima, posteriorly and anteriorly overlying the large plaque. Within the central portion of the latter, lipid droplets lay between the fusiform spaces and were largely in extracellular situations.

Refractile material, as demonstrated with polarized light, varied in its distribution (Fig. 31). That in the thickened intima appeared as rods or flat rectangular plates. In the central portion of the large anterior plaque, there were many long needle-like crystals which at times were segmented. In one bird (15-89) the anterior plaque resembled more closely the spontaneous fibrous lesion, though lipid-containing foam cells of the fibroblastic type were seen at its lateral angles.

Relation of the Lipids Deposited in the Aorta to Those in Plasma

The concentrations of cholesterol, total fatty acids, and phospholipids found in the plasma of control, cholesterol-fed, and stilbestrol-injected birds are recorded in Tables IV-VI. They show that the 2 latter groups of birds differed not only in the total amounts of lipids contained in their plasma but also in the composition of these lipids (*i.e.*, proportions of cholesterol, triglycerides, and phospholipids present). Thus in the stilbestrol-treated birds, values for total lipids well above 10,000 mg. per cent were not uncommon—the highest value was 17,700 mg.—, whereas in the cholesterol-fed birds the values for total lipids did not exceed 5000 mg. Although the concentration of plasma cholesterol was in most cases higher in the stilbestrol-treated birds than in those fed cholesterol, this lipid constituent did not account for the difference in the total lipids observed in the 2 groups. It was the triglycerides and phospholipid components that accounted for the amazingly high concentrations of lipids in the plasma of the stilbestrol-treated birds. Values for phospholipids between 3000 and 5000 mg. and values for neutral fat in the neighborhood of 9 to 10,000 mg. per 100 cc. of plasma were not uncommonly found in the estrogen-injected birds.

In those fed cholesterol, on the other hand, the highest concentrations of phospholipids and neutral fat were respectively 645 and 3900 mg.

As judged from reactions to stains and from examination with polarized light, the composition of the lipids deposited in the arterial walls differed in the cholesterol-fed and stilbestrol-injected birds. In the aorta of the former, cholesterol constituted the bulk of the lipids deposited, whereas in the stilbestrol-treated birds lipids other than cholesterol predominated. This finding is somewhat

TABLE IV
Plasma Lipids of Control (Untreated) Male Birds
(All lipid values are expressed as mg. per 100 cc.)

Bird	Interval after start of experiment	Cholesterol			Total fatty acids	Phospho-lipids	Total lipids
		Total	Free	Ester			
15-02	mor.						
	6.5	134	44	90	227	187	361
15-27	0	107	16	91	280	183	387
	1.5	88	22	66	310	171	398
	3.0	105	25	80	352	126	457
15-79	6.5	86	30	46	282	125	368
15-81	0	92	25	67	380	223	372
	1.5	81	34	47	315	157	396
	3.0	101	29	75	380	158	484
15-82	6.5	67	22	45	253	137	321
15-88	6.5	107	32	85	272	189	379
15-90	6.5	102	35	67	288	176	390
15-91	6.5	99	27	72	257	148	356
15-99	6.5	55	19	36	267	118	322

surprising, for, as noted above, the concentration of cholesterol in plasma of the estrogen-treated birds was as great as, and sometimes greater than, that of the cholesterol-fed birds. The excessive amounts of phospholipids and triglycerides contained in the plasma of the estrogen-treated birds would appear to have influenced cholesterol deposition in the aortas. The extent to which plasma cholesterol accumulates in the aorta may depend not only on the absolute concentration of cholesterol in plasma, but also on the relative proportions of the various lipid constituents present in plasma. In other words, cholesterol may compete with other plasma lipids for space in the arterial wall.

TABLE V
Plasma Lipids of Stilbestrol-Injected Male Birds
 (All lipid values are expressed as mg. per 100 cc.)

Bird	Interval after first injection	Cholesterol			Total fatty acids	Phospho-lipids	Total lipids
		Total	Free	Ester			
	<i>mos.</i>						
15-08	1	114	26	88	508	276	622
	2.5	741	557	184	10900	4300	10700
	4.5	694	481	213	10100	3000	10700
	6.5	444	307	137	7270	2200	7710
15-10	2.5	575	518	257	11300	3100	11800
	4.5	758	532	226	10500	3180	11200
	6.5	532	316	216	12400	3200	12900
15-24	1	234	67	167	939	537	1170
15-26	1	99	52	47	1340	438	1440
	2.5	570	314	256	6400	3200	7020
	4.5	920	535	385	11600	3240	12000
	6.5	119	37	82	272	204	391
15-30	1	214	41	173	755	360	969
	2.5	780	605	175	9300	3100	10100
	4.5	480	440	40	12900	4180	13300
15-35	1	129	38	91	597	295	726
	2.5	327	129	198	2190	580	2510
15-36	1	172	56	116	597	141	769
	2.5	585	368	217	14400	3330	14900
	3.0	735	222	513	16900	4930	17600
15-45	1	116	45	71	382	205	498
	2.5	96	34	62	387	197	487
	4.5	762	620	142	10900	2680	11600
	6.5	515	312	203	11400	2830	11900
15-48	1	218	72	146	925	546	1140
	2.5	511	195	316	2430	758	2940
	4.5	885	510	375	9680	2840	10500
	6.5	381	232	149	4320	1370	4700
15-58	0	180	64	116	680	428	860
	1.5	341	230	111	3080	977	3420
15-65	0	101	39	62	497	302	598
	1.5	440	302	138	6050	1970	6490
	2.5	296	207	89	3030	1070	3320
	6.5	99	31	68	710	250	899
15-85	1	151	47	104	386	106	537
	2.5	565	368	197	13900	3300	14400
	3.0	615	224	391	17100	5200	17700

TABLE VI
Plasma Lipids of Cholesterol-Fed Male Birds
 (All lipid values expressed as mg. per 100 cc.)

Bird	Period fed cholesterol	Cholesterol			Total fatty acids	Phospho- lipids	Total lipids
		Total	Free	Ester			
	<i>mos.</i>						
15-07	1	400	98	302	610	215	1010
	1.5	322	70	252	560	197	882
	2.5	251	101	150	768	253	1010
	3.0	271	108	163	819	294	1090
	6.5	403	296	107	2240	505	2640
15-18	1	185	81	104	1140	238	1330
	2.5	230	147	83	1200	383	1430
	5.5	172	146	26	1870	537	2040
15-19	1	227	139	88	3270	518	3500
	2.5	226	138	88	2730	492	2960
15-38	1	184	82	102	2650	485	2830
	2.5	590	318	272	2500	380	3090
	3.0	775	238	537	3330	517	4100
15-59	2.5	236	114	122	2380	415	2620
	6.5	392	306	86	4340	575	4730
15-60	1	251	148	103	2610	518	2860
	2.5	425	191	234	3520	592	3940
	4.5	158	139	19	2480	645	2650
15-67	2.5	255	129	126	3020	387	3270
15-72	1.5	566	340	226	2280	369	2840
	3.0	387	338	40	2720	335	3090
	4.5	458	247	211	3160	558	3620
	6.5	555	340	215	2300	404	2850
15-84	2.5	264	110	154	595	545	859
	6.5	154	98	56	468	547	722
15-86	1	187	79	108	1210	216	1400
	2.5	234	110	124	1720	332	1960
	4.5	338	130	208	3450	376	3970
	6.5	377	290	87	3050	440	3430
15-89	2.5	175	53	122	287	120	462
	6.5	254	135	119	2520	217	2770
16-00	1	357	177	180	1590	225	1940
	2.5	560	360	200	2030	356	2590
	3.0	720	318	402	3260	517	3980

It is of interest to note that the plasma of the stilbestrol-treated birds was milky, opaque, and of the same lemon yellow color as the intimal lesions in the aortas of these birds. The plasma of the cholesterol-fed birds, however, was tan-colored and resembled the color seen in their intimal lesions.

DISCUSSION

Three forms of arterial disease in the bird are described here: the spontaneously occurring one, that induced by stilbestrol injections, and that brought about by cholesterol feeding. These 3 lesions are fundamentally similar in nature. In the thoracic aorta, all 3 are characterized by lipid deposition within the intima and the adjacent media. They differ, however, in 2 important aspects: (1) the degree of lipid infiltration and (2) the proportion of the various lipids that infiltrate the wall of the artery.

When present, the spontaneous *thoracic* lesion of the rooster was visible only by microscopic examination and consisted entirely of a lipid infiltration of the intima, none of which was identifiable as cholesterol. The degree of lipid infiltration in the intima and adjacent media of the thoracic aorta induced by stilbestrol and cholesterol feeding was great enough to produce grossly visible lesions which were accompanied by fibrous proliferation. Although cholesterol was identified in both of the experimentally induced thoracic lesions, the bulk of the lipid in the stilbestrol-induced lesion was not cholesterol, whereas in the cholesterol-fed bird cholesterol formed the bulk of the lipid infiltration.

The basic *abdominal* lesion that occurs in all 3 types is fibrous in nature and appears finally as an elongated longitudinal plaque. In the untreated birds the plaque contained small amounts of lipid material, some of which was cholesterol. In the stilbestrol-treated birds the spontaneous fibrous plaque was heavily infiltrated with lipids, part of which was cholesterol. In those fed cholesterol, the spontaneous plaque was greatly modified by a heavy deposition of cholesterol.

In both the cholesterol-fed and the stilbestrol-treated birds, more lipids were deposited in the spontaneous fibrous plaque than in the neighboring normal vascular tissue. A similar phenomenon is found in man suffering from syphilitic aortitis; atheromatous deposition is most pronounced in those portions of the aorta which have been altered by the syphilitic fibrosing process. These findings suggest that the fibrotic intima is more permeable to lipids than the normal.

Two types of spontaneous arteriosclerosis are found in the bird, and, according to Dauber (12), one of these, namely the lipid infiltration of the intima of the thoracic aorta, occurs only in the female bird. A minimal degree of lipid infiltration, however, was found also in the thoracic aortas of our control group of roosters. This same thoracic lipid lesion was also observed by us in several roosters and capons over 5 years of age. Thus, while this lesion occurs in both roosters and hens, it is obviously more extensive in hens, probably as a result of

the periodic lipemia associated with egg laying (1). With the artificially induced lipemia of cholesterol feeding or stilbestrol implantation, this lipid infiltration of the thoracic aorta of the rooster is accentuated, and, when extensive enough, is associated with proliferation of fibroblastic foam cells. This process tends to involve the entire aorta, but is most striking in the thoracic portion.

The second type of spontaneous arteriosclerosis seen in the bird is the ridge-like intimal thickening of the abdominal aorta. This occurs in both the hen and the rooster, but in the former, according to Dauber (12), lipid is more abundant, again probably the result of the physiological lipemia associated with egg laying. The etiology of this fibrotic lesion is still obscure. In both sexes the lesion is fundamentally a fibrosing process in which lipid material gradually accumulates, so that greater amounts are seen in older birds. Prolonged lipemia produced by cholesterol feeding or by stilbestrol implantation modifies this lesion by depositing in it excessive amounts of lipids.

The lesion produced by stilbestrol implantation more nearly resembled the spontaneous lesion than did the one occurring after cholesterol feeding. The cholesterol-induced lesion is striking because of its high content of cholesterol crystals. This type of arterial lesion should be considered part of a widespread cholesterol storage process and therefore in the nature of an artificial arterial disease.

The results presented here clearly establish that a sustained hyperlipemia, whether of endogenous origin (stilbestrol-injected birds) or of exogenous origin (cholesterol-fed birds), can result in an infiltration of lipids in the aortic wall. While cholesterol forms a striking component of the experimentally induced atherosclerotic lesions studied here, it does so mainly because of its insoluble nature. It would appear that lipids other than cholesterol also infiltrate the vascular wall, but these can subsequently diffuse out of the arterial wall. Cholesterol, on the other hand, after its deposition in the arterial wall, makes its appearance finally as large crystalline masses which are no longer movable.

The late fibrotic *abdominal* plaque in the bird, particularly after modification by deposition of large amounts of cholesterol and other lipids, resembles the late atherosclerotic lesion found in man, for both are fibrous plaques containing cholesterol and other lipids. In the normal male bird, however, the large abdominal aortic plaque is present long before lipid deposits become abundant, whereas in man the lipid deposit precedes fibrous scarring. It is evident therefore that, despite their similarity in appearance, the mechanism resulting in the formation of the abdominal lesion in the bird differs from that which produces human atherosclerosis.

SUMMARY

A new experimental procedure for the production of arteriosclerosis in the bird is described. The subcutaneous implantation of diethylstilbestrol by

means of which a sustained increase in the concentration of cholesterol, phospholipid, and neutral fat can be readily established, is shown to induce atherosclerosis of the aorta.

The atherosclerosis has been compared with that artificially induced in the bird by the prolonged feeding of cholesterol and also with that occurring spontaneously. The stilbestrol-induced lesion more closely resembled the spontaneously occurring one in the bird than did that produced by cholesterol feeding. But all 3 lesions were fundamentally similar, differing only in the amounts and proportions of the various lipid constituents present.

The concentrations of cholesterol in plasma of the stilbestrol-treated and cholesterol-fed birds were of the same order. Yet cholesterol constituted a greater proportion of the lipids deposited in the arterial wall of the cholesterol-fed than in that of the stilbestrol-treated birds. This finding suggests that the cholesterol content of the vascular lesion depends not only on the absolute concentration of cholesterol in plasma, but also on the proportion of cholesterol to other lipid constituents in plasma.

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EXPLANATION OF PLATES

Control Group

PLATE 17

FIG. 1. Bird 15-28. Thoracic aorta showing minimal lipid infiltration of intima and adjacent media. Sudan IV and hematoxylin stain. $\times 78$.

FIG. 2. Bird 15-90. Thoracic aorta showing absence of lipid deposition. Sudan IV and hematoxylin stain. $\times 78$.

FIG. 3. Bird 15-02. Abdominal aorta showing minimal intimal thickening without lipid infiltration. Sudan IV and hematoxylin stain. $\times 78$.

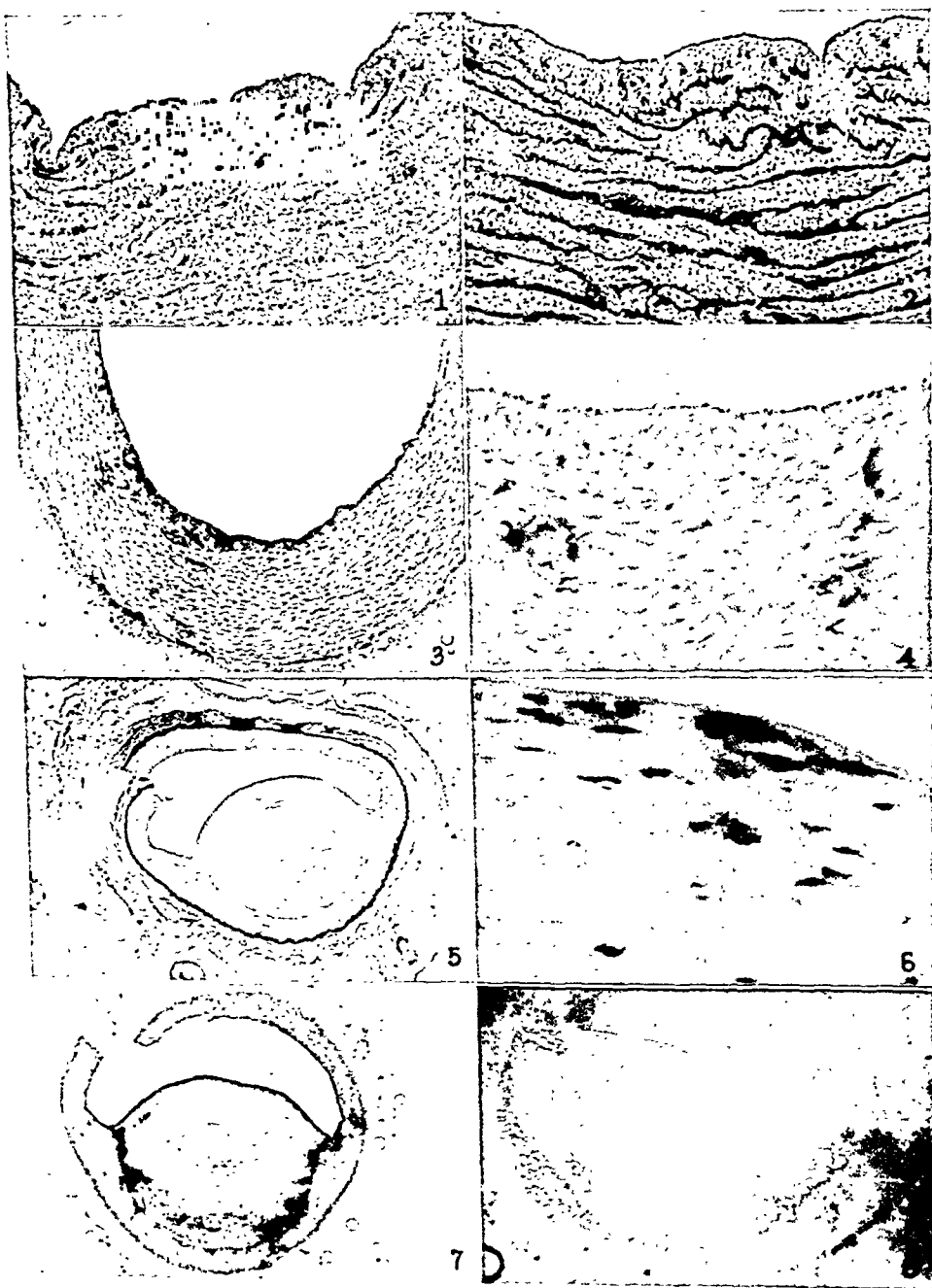
FIG. 4. Bird 15-02. Abdominal aorta showing early intimal fibrous thickening. Hematoxylin and eosin stain. $\times 210$.

FIG. 5. Bird 15-90. Abdominal aorta showing large fibrous abdominal plaque. Verhoeff-van Gieson stain. $\times 23$.

FIG. 6. Bird 15-90. Abdominal aorta showing transverse arrangement of cells and fibers of the large abdominal plaque. Hematoxylin and eosin stain. $\times 820$.

FIG. 7. Bird 15-90. Abdominal aorta showing large intimal plaque with lipid deposition in the deeper portions. Sudan IV and hematoxylin stain. $\times 23$.

FIG. 8. Bird 15-91. Abdominal aorta showing large intimal plaque with cholesterol in the sites of lipid deposition. Polarized light. $\times 35$.



(Chaikoff *et al.*: Atheromatosis in aorta of bird)

Stilbestrol Group

PLATE 18

FIG. 9. Bird 15-26. Thoracic aorta showing intimal thickening. Hematoxylin and eosin stain. $\times 78$.

FIG. 10. Bird 15-26. Thoracic aorta showing lipid infiltration of the intima and adjacent media. Sudan IV and hematoxylin stain. $\times 78$.

FIG. 11. Bird 15-26. Thoracic aorta showing intimal thickening with vertical arrangement of reticulum fibers. Laidlaw stain. $\times 78$.

FIG. 12. Bird 15-26. Thoracic aorta intima showing lipid droplets in connective tissue cells. Sudan IV and hematoxylin stain. $\times 210$.

FIG. 13. Bird 15-26. Thoracic aorta showing deposition of cholesterol in intima and adjacent media. Polarized light. $\times 35$.

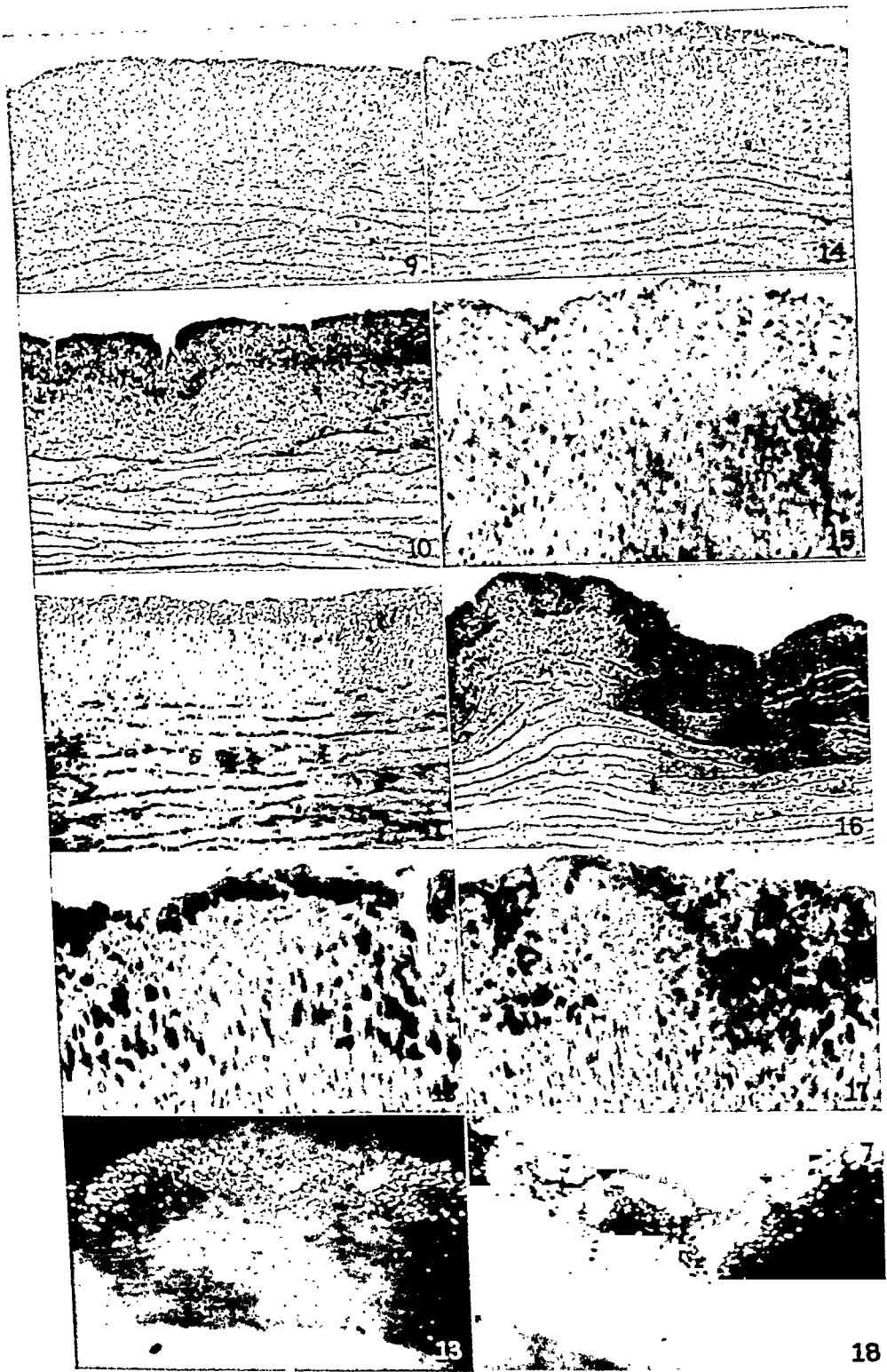
FIG. 14. Bird 15-48. Thoracic aorta showing intimal thickening. Hematoxylin and eosin stain. $\times 78$.

FIG. 15. Bird 15-48. Thoracic aorta showing vacuolated connective tissue cells of thickened intima. Hematoxylin and eosin stain. $\times 78$.

FIG. 16. Bird 15-48. Thoracic aorta showing extensive lipid deposits in the intima and adjacent media. Sudan IV and hematoxylin stain. $\times 78$.

FIG. 17. Bird 15-48. Thoracic aortic intima showing lipid droplets in connective tissue cells. Sudan IV and hematoxylin stain. $\times 210$.

FIG. 18. Bird 15-48. Thoracic aorta showing deposition of cholesterol in intima and adjacent media. Polarized light. $\times 35$.



Stilbestrol Group

PLATE 19

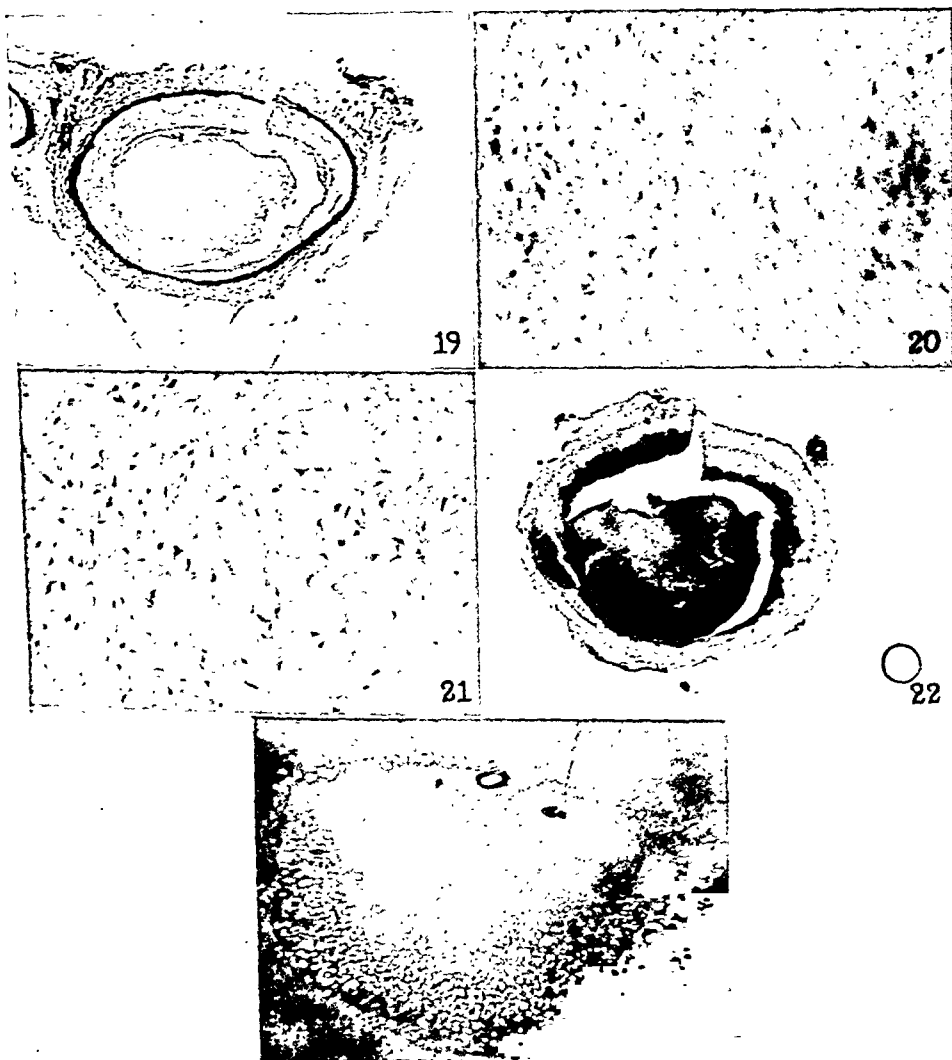
FIG. 19. Bird 15-65. Abdominal aorta showing large fibrous abdominal plaque. Verhoeff-van Gieson stain. $\times 23$.

FIG. 20. Bird 15-65. Abdominal aortic plaque showing mild vacuolization of connective tissue cells. Hematoxylin and eosin stain. $\times 210$.

FIG. 21. Bird 15-65. Abdominal aortic plaque showing marked vacuolization of connective tissue cells. Hematoxylin and eosin stain. $\times 210$.

FIG. 22. Bird 15-65. Abdominal aorta showing large intimal plaque with lipid deposition in plaque and remainder of vascular wall. Sudan IV and hematoxylin stain. $\times 23$. (Compare with Fig. 7.)

FIG. 23. Bird 15-65. Abdominal aorta showing large intimal plaque with cholesterol in sites of lipid deposition. Polarized light. $\times 35$. (Compare with Fig. 8.)



(Chaikoff *et al.*: Atheromatosis in aorta of bird)

Cholesterol Group

PLATE 20

FIG. 24. Bird 15-84. Thoracic aorta showing intimal thickening. Hematoxylin and eosin stain. $\times 78$.

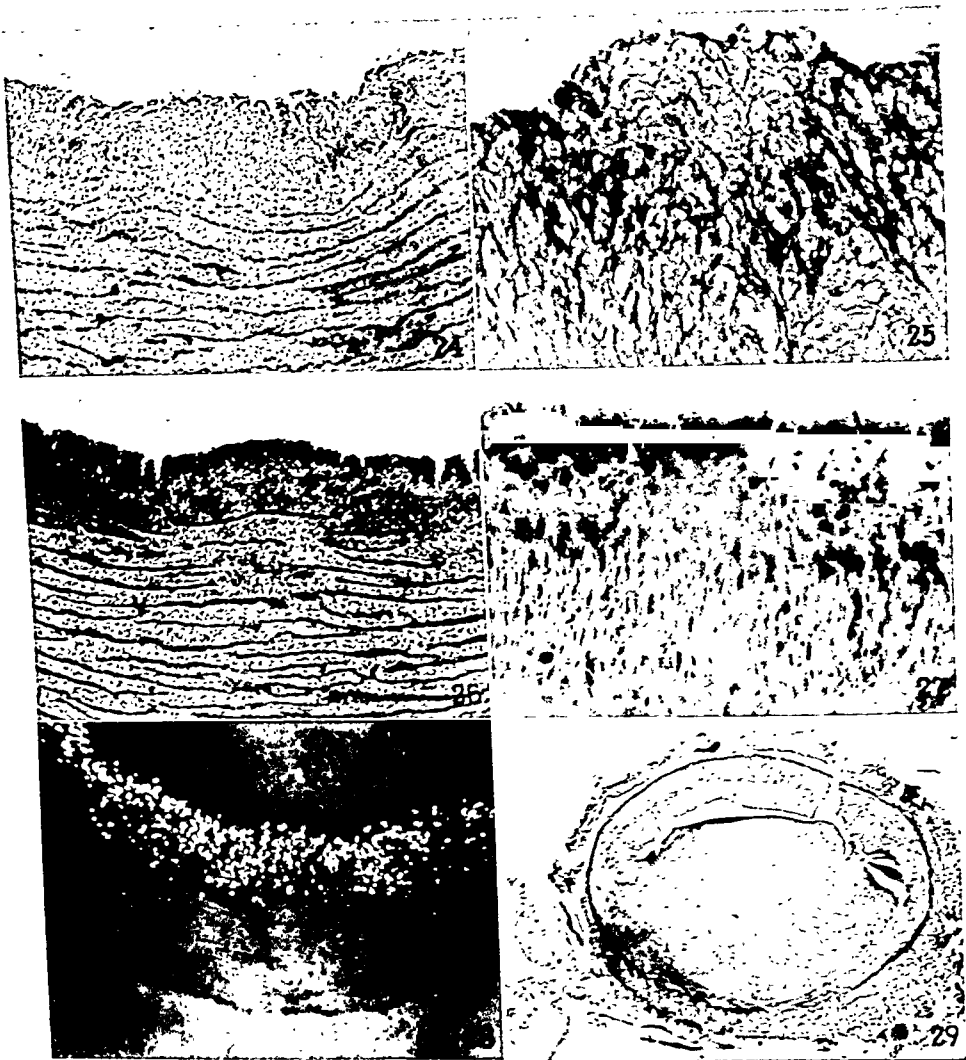
FIG. 25. Bird 15-72. Thoracic aortic intima showing perpendicular arrangement of reticulum fibers. Laidlaw stain. $\times 210$.

FIG. 26. Bird 15-26. Thoracic aorta showing lipid infiltration of the intima and adjacent media. Sudan IV and hematoxylin stain. $\times 78$.

FIG. 27. Bird 15-84. Thoracic aortic intima showing lipid droplets in connective tissue foam cells. Sudan IV and hematoxylin stain. $\times 210$.

FIG. 28. Bird 15-18. Thoracic aorta showing deposition of cholesterol in intima and adjacent media. Polarized light. $\times 35$. (Compare with Figs. 13 and 18.)

FIG. 29. Bird 15-07. Abdominal aorta showing large fibrous plaque and extensive intimal thickening. Verhoeff-van Gieson stain. $\times 23$. (Compare with Figs. 5 and 19.)



(Chaikoff *et al.*: Atheromatosis in aorta of bird)

Cholesterol Group

PLATE 21

FIG. 30. Bird 15-07. Abdominal aorta showing large intimal plaque with lipid deposition in plaque and remainder of vascular wall. Sudan IV and hematoxylin stain. $\times 23$. (Compare with Figs. 7 and 22.)

FIG. 31. Bird 15-07. Abdominal aorta showing large intimal plaque with abundant cholesterol in sites of lipid deposition. Polarized light. $\times 35$. (Compare with Figs. 8 and 23.)

FIG. 32. Bird 15-84. Abdominal aorta showing large fibrous plaque and intimal thickening. Verhoeff-van Gieson stain. $\times 23$. (Compare with Figs. 5 and 19.)

FIG. 33. Bird 15-84. Deeper portion of abdominal plaque showing vesicular fibroblasts. Hematoxylin and eosin stain. $\times 210$.

FIG. 34. Bird 15-84. Superficial portion of plaque showing collections of macrophages. Hematoxylin and eosin stain. $\times 210$.

FIG. 35. Bird 15-84. Abdominal plaque showing cholesterol clefts and calcific deposits. Hematoxylin and eosin stain. $\times 210$.



(Chaikoff *et al.*: Atheromatosis in aorta of bird)

HYPERTENSIN IN THE SYSTEMIC BLOOD OF ANIMALS WITH
EXPERIMENTAL RENAL HYPERTENSIONBy FRANK GOLLAN, M.D., EVELYN RICHARDSON, AND
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The final proof for the correctness of the present view that experimental renal hypertension is caused by a humoral mechanism of renal origin would be the demonstration of the presence of a pressor substance of renal origin in the circulating systemic blood of animals with persistent hypertension of this type. The proof that essential human hypertension is of similar origin would be the demonstration of the corresponding substance in the blood of human beings with this type of hypertension.

Although there are many reasons for assuming that *in vivo* the proteolytic action of the enzyme renin on a pseudoglobulin (hypertensinogen) in the plasma results in the formation of the vasoconstrictor substance hypertensin, a polypeptide, nevertheless the final proof for the existence of this humoral mechanism in the circulating systemic blood of animals with persistent, benign, experimental renal hypertension has been lacking until now, and some investigators have asserted that in the later stages a neurogenic factor is responsible for the persistence of the elevated blood pressure. The present publication deals with the demonstration and identification of a pressor substance (hypertensin) in the systemic blood of animals in the earlier period of experimental renal hypertension.

The development of the method for the detection of hypertensin in the blood was based on the finding of Bean (1) that the enzyme hypertensinase, which destroys hypertensin, is almost without activity at 0°C. By the elimination of hypertensinase activity immediately after the withdrawal of the systemic blood, and the immediate separation and precipitation of the plasma, it should be possible to detect any hypertensin present in the circulating blood at the time the specimen of blood is withdrawn. Furthermore, it has been shown by Sapirostein and collaborators (2) that at 0°C. renin continues to act on renin substrate, although at a diminished rate, and that the reaction reaches equilibrium in about 2 hours. Thus, by prolonged incubation of the plasma, in the cold, the action of a small amount of renin on the renin substrate in the blood can be enhanced, *in vitro*, and the presence of renin detected without the addition of renin substrate.

Method

With 50 cc. syringes containing heparin solution, 200 cc. of blood was drawn rapidly from the jugular vein or femoral artery of a dog weighing 12 to 15 kilos. An 18 gauge needle was used, so that the withdrawal of the entire amount was completed in less than 5 minutes. Every syringe of blood was immediately chilled by being emptied into a 250 cc. centrifuge cup standing in an acetone and dry ice bath, at -20°C . During the process of cooling, the blood was stirred constantly by a bent glass rod attached to an air-driven stirrer. When the entire amount of blood had been put into the centrifuge cup, no more dry ice was added to the bath, the temperature of which was usually about -10°C . when that of the blood was about 5°C . The entire 200 cc. of blood was cooled to 0°C . in not more than 10 minutes. The moment the blood had reached 0°C ., the stirring was stopped and the centrifuge bottle containing the blood was removed instantly from the ice bath in order to avoid freezing of the blood. The blood was then centrifuged at 0°C . for 30 minutes at 15,000 R.P.M. in a brine-cooled centrifuge. After centrifugation the plasma usually had a temperature of about 7°C . The plasma, which was not hemolyzed, if freezing had been avoided, was drawn off by means of a previously cooled syringe, transferred to a cooled 250 cc. centrifuge bottle, and left standing in a cooler at 0°C . for 24 hours. Two hours at 0°C . was the minimum incubation time for the optimum yield of hypertensin. At the end of that time the plasma was transferred to a 250 cc. beaker previously cooled in a deep freezer at -20°C . The beaker was placed in a dish containing a dry ice and acetone mixture at -10°C . The pH of the plasma was now adjusted to 4.5 with 10 N HCl solution by use of glass electrodes. About one-fourth of the cold plasma was poured into another 250 cc. beaker previously heated in a bath of boiling water. The plasma was stirred constantly while it was being coagulated, and, when the temperature had reached 70°C ., another fourth of the cold plasma was added. The heating was continued until the temperature of the plasma had reached at least 90°C . In this way the hypertensinase of the first fourth of the plasma acted at body temperature for a very short time. At pH 4.5 a clear supernatant fluid resulted after heat coagulation of the plasma proteins. This liquid was filtered off with suction through a small Büchner funnel. The pH of the clear filtrate was usually about 5.5 and was then adjusted to 7.4 with N/10 NaOH solution. The final volume, approximately one-fourth of the original amount of blood, or about 50 per cent of the plasma, was cooled to body temperature and injected intravenously into a trained unanesthetized dog. A 100 cc. syringe and an 18 gauge needle were used for the injection, which lasted approximately 30 seconds.

EXPERIMENTS

The Demonstration of Hypertensin in the Systemic Blood of Normal Dogs after the Intravenous Injection of Various Quantities of Renin.—It was demonstrated first that the intravenous injection of the solution prepared in the manner described above from a large quantity of the serum of an animal with normal blood pressure has no pressor effect. As a control of the experiments summarized in Table II, samples of 200 cc. of systemic blood of normal dogs that had not received an intravenous injection of renin were treated by the method described above and tested for the presence of renin and hypertensin. Table I shows that the plasma from this quantity of blood of normal dogs did not contain or develop a pressor substance in an amount detectable by the method used.

The next step was to demonstrate that the presence of renin in the circulating

blood can be detected. Since it is assumed that renin is the substance which initiates the humoral mechanism of experimental renal hypertension, the attempt was made to learn whether the result of the interaction of renin and renin substrate (hypertensinogen) *in vivo*, as well as *in vitro*, is hypertensin, which can be recovered from the circulating blood. For this purpose various quantities of standardized renin (3), of known potency, were injected into normal dogs, and the withdrawal of 200 cc. of venous blood was begun at the height of the rise of blood pressure, 2 to 3 minutes after the injection of the renin was completed. The blood was then treated in the manner described above for the demonstration of hypertensin. To demonstrate existent hypertensin, some of the specimens were coagulated by heat immediately after the separation of the plasma. In order to determine how much hypertensin would be formed by the amount of renin and hypertensinogen present in the sample,

TABLE I
Hypertensin in Systemic Blood of Normal Dogs

Dog. No.	Plasma from 200 cc. of blood (incubation at 0°C.).	Rise in blood pressure (direct mean femoral)
	<i>hrs.</i>	<i>mm.</i>
8-43	24 hrs.	0
10-49	" "	0
10-48	" "	0
10-47	" "	0
10-45	" "	0
10-48	" "	0

other specimens were allowed to stand at 0°C. for 24 hours before they were coagulated. The results obtained from the intravenous injection of 1 to 50 units of renin are shown in Table II. They confirm the finding of Houssay, Braun-Menendez, and Dexter (3 *a*) that intravenously injected renin can be detected in the systemic blood.

Table II shows that immediately after the intravenous injection of renin a non-protein, heat-stabile, vasopressor substance is rapidly formed, which can be isolated from the plasma, and which, when injected intravenously into a normal dog, produces a type of rise of blood pressure which is characteristic of hypertensin, *viz.* an immediate rise, which reaches a maximum in 1 minute or less, and is of short duration, 3 minutes or less. Thus, it has been shown that the result of the action of renin on the substrate in the plasma *in vivo* is the same as that which occurs *in vitro* (4). Table II shows that the amount of hypertensin found in the plasma during the first 5 minutes after the injection of renin increases with the amount of renin injected into the blood and that less failures of detection of hypertensin in the blood occur when larger amounts

of renin are injected. Even after the intravenous injection of only 1 unit of renin there is some formation of hypertensin, amounting to as much as half a unit in 200 cc. of blood, if the plasma is coagulated immediately after separa-

TABLE II

Dog No.	Weight	Units of renin injected intravenously	Plasma from 200 cc. of blood (kept at 0°C.)	Rise in blood pressure (direct mean femoral)
	lbs.			mm.
8-72	39	1	—	0
10-44	22	1	—	0
9-33	36	1	—	15
10-24	29	1	—	15
10-40	19	1	—	10
9-32	47	1	24 hrs.	0*
10-62	30	1	" "	30
9-32	47	1	" "	25
10-35	26	5	—	30
10-14	27	5	—	0*
9-32	47	5	—	30
10-14	27	5	—	0*
10-41	23	5	—	30
10-14	27	5	—	10
8-48	23	5	—	40
8-43	40	5	—	10
8-82	29	5	—	30
10-10	42	5	—	40
10-29	30	5	24 hrs.	50
9-43	19	5	" "	50
10-27	28	10	—	0*
9-95	32	10	—	30
3-31	48	10	—	0*
10-10	42	10	—	40
9-87	20	10	—	40
9-32	33	20	—	30
10-19	47	20	—	30
9-43	25	20	24 hrs.	60
10-36	31	50	" "	75

* These failures to demonstrate any pressor effect, and also the variations, are probably due to technical errors, or to destruction of the hypertensin during preparation.

tion, and about 1 unit if the plasma is kept at 0°C. for 24 hours before it is coagulated by heat.

The Demonstration of Hypertensin in the Systemic Blood of Dogs with Benign Experimental Renal Hypertension.—For this series of experiments dogs were made hypertensive by constriction of their main renal arteries (5). In some dogs this operation was performed unilaterally, but in most of the animals

both renal arteries were constricted and, after the blood pressure had risen to a hypertensive level, blood was withdrawn from the jugular vein and treated in the manner described above for the demonstration of hypertensin.

TABLE III

(a) Hypertensin in plasma of dogs with experimental hypertension after unilateral clamping of the main renal artery.

Dog No.	Weight	Period of hypertension	Blood pressure	Amount of blood	Incubation at 0°C.	Rise in blood pressure
	<i>lbs.</i>	<i>days</i>	<i>mm. Hg.</i>	<i>cc.</i>		<i>mm.</i>
10-30	20	3	175	190	—	0
10-39	30	4	180	400	24 hrs.	25
10-24	29	11	165	400	" "	30
10-39	30	20	185	400*	" "	0†

(b) Hypertensin in plasma of dogs with experimental hypertension after bilateral clamping of the main renal arteries.

Dog No.	Weight	Period of hypertension	Blood pressure	Amount of blood	Incubation at 0°C.	Rise in blood pressure
	<i>lbs.</i>		<i>mm. Hg</i>	<i>cc.</i>		<i>mm.</i>
10-45	23	2 days	125	300	—	15
10-39	30	3 "	190	70	—	5
10-27	26	5 "	140	225	24 hrs.	25
10-40	22	" "	140	300	" "	0
10-30	20	11 "	190	450	" "	15
10-30	20	19 "	180	330	" "	40
10-47	28	23 "	175	360	" "	30
9-22	24	25 "	200	400	" "	0
10-29	33	79 "	190	200	—	10
10-29	33	89 "	190	200	24 "	0
10-29	33	3 mos.	190	520	" "	20
8-67	38	18 "	190	200	—	15
4-61	37	3 yrs.	200	200	—	0

* The plasma filtrate, after precipitation, was concentrated to 17 cc. by pervaporation.

† Some of the failures to demonstrate a pressor effect, and also the variations, may have been the result of technical errors or destruction of the hypertensin during preparation.

After unilateral or bilateral constriction of the renal arteries, the pressor substance was detected in the systemic blood of some of the dogs with experimental benign renal hypertension that had existed for as long as 3 months (see Table III), especially when large amounts of blood were tested.

The amount of blood necessary for the detection of half to 1 unit of the pressor substance (about 200 cc.) is equal to about one-fifth of the whole blood volume of the animal. In control tests it was noticed that when an unusually large amount (500 cc.) of blood was drawn from single normal

animals, the plasma supernatant (after heat coagulation of the plasma) also gave a slight pressor effect, up to half a unit of hypertensin in 350 to 500 cc. blood. To eliminate this possibility of misinterpretation of the results, samples of not more than 200 cc. of blood were taken from each of several normal and hypertensive dogs and pooled. Each large sample was treated in the manner previously described for the demonstration of hypertensin and the final products were pooled and concentrated by pervaporation in front of a revolving fan, at room temperature, in a 2 inch cellophane tubing, from about 400 cc. to 50 cc. or less. Control tests with known amounts of diluted hypertensin showed that by this method of concentration about one-third of the hypertensin activity is lost.

TABLE IV
Hypertensin in Large Pooled Samples of Blood

Normal dogs			
Amount of pooled systemic blood	Incubation at 0°C.	Treated plasma concentrated to cc.	Rise in blood pressure (direct mean femoral)
cc.			mm.
800	24 hrs.	25	0
800	" "	25	0
700	" "	35	0
1500	" "	50	0
Hypertensive dogs			
700	24 hrs.	35	30
1500	" "	50	45
800	72 "	17	30

Table IV shows that in chronic benign experimental hypertension, hypertensin can be demonstrated in the circulating blood, if large amounts of systemic blood pooled from several animals are used.

The Demonstration of Hypertensin in Blood from the Renal Vein of an Ischemic Kidney.—It has been shown that by the addition of renin substrate to the renal vein blood from an ischemic kidney, and incubation, hypertensin can be demonstrated (6), although none can be detected without the addition of the hypertensinogen. The addition of the latter presumably allows the renin present in the blood to form more hypertensin. Yet it seemed important to demonstrate the formation of hypertensin from the renin and hypertensinogen present in the renal venous blood from an ischemic kidney, by the method described in this paper.

Table V shows that it was possible to demonstrate the existence of hypertensin and, by inference, renin, in the renal vein blood from a kidney the renal

artery of which was constricted. Renal vein blood from a normal kidney did not contain hypertensin.

In normal dogs, one renal artery was constricted by a clamp. After 4 days, when the blood pressure had risen 40 to 70 mm. Hg above normal, the dogs were anesthetized with ether and, by retroperitoneal approach, renal vein blood from the normal kidney as well as from the kidney with renal artery constricted was taken by separate rapid cannulation of both veins. The bloods were treated in the usual manner, immediately after withdrawal, and also after an incubation period of 24 hours at 0°C.

The Demonstration of Hypertensin in the Systemic Blood of Dogs with Experimental Renal Hypertension of the Malignant Type.—Both main renal arteries of dogs were constricted so that the malignant type of hypertension (7), with renal insufficiency and necrotizing arteriolitis, resulted.

TABLE V
Hypertensin in Blood from Renal Vein

	Amount of blood	Incubation of plasma at 0°C.	Rise in blood pressure (direct mean femoral)
	cc.		mm.
Normal renal vein blood.....	125*	—	0
" " " "	145*	24 hrs.	0
Ischemic renal vein blood.....	200	—	20
" " " "	160	24 hrs.	45

* The amount of normal blood was less, but there was not sufficient difference to account for the difference in content of pressor substance.

Table VI shows that in the malignant phase of experimental renal hypertension the existence of relatively large amounts of hypertensin in the systemic blood can be demonstrated. In 300 cc. of systemic blood of dog 10-34, weighing 20 pounds, 6 units of hypertensin were detected, after incubation for 24 hours.

The Effect of Hypertensinase on the Pressor Substance Derived from the Systemic Blood of a Normal Dog, after the Intravenous Injection of Renin, and from the Systemic Blood of a Dog with Malignant Hypertension.—If the pressor substance present in the blood of animals with experimental renal hypertension is really hypertensin, it should be destroyed by hypertensinase.

Control experiments were performed first, of which the following are examples.

Twenty units of hog renin were injected intravenously into a normal dog, No. 9-43, and, after 2 minutes, 200 cc. of blood was withdrawn from the jugular vein. The blood was chilled immediately to 0°C. and centrifuged for 1 hour in the cold. The plasma was then separated and left standing at 0°C., for 24 hours. The 120 cc. of plasma was then divided

into two equal parts. The first part was immediately coagulated by heat, after adjustment of pH to 4.5. The second part was first subjected to the action of the hypertensinase in the blood by incubation at 40°C. for 24 hours and then also coagulated by heat, after adjustment of pH to 4.5. Both samples were filtered through Büchner filters, and the pH of the filtrates was adjusted to 7.2. Then the solutions were injected into normal dogs to test for the hyper-

TABLE VI

Hypertensin in Blood of Dogs with Experimental Malignant Renal Hypertension (Both Renal Arteries Greatly Constricted)

Dog No.	Weight	Period of hypertension	Blood pressure	Amount of blood withdrawn	Incubation	Total yield of deproteinized plasma	Amount injected to test for pressor effect	Rise in blood pressure (direct mean femoral)
	lbs.	days	mm.	cc.	hrs.	cc.	cc.	mm.
10-39	30	3	220	500	48	200	20	25
9-98	31	2	175	850	48	340	70	30
10-46	29	3	185	70	—	30	30	15
10-30*	21	3	185	190	—	80	80	0
10-45	23	2	205	300	—	120	60	40
							60	50
10-34	23.5	2	175	290	24	120	20	30

* Dog 10-30 was the only one that was not uremic when the sample of blood was taken, but that azotemia alone is not accompanied by the accumulation of renin or hypertensin in the blood is shown by the absence of a pressor substance in the blood of two bilaterally nephrectomized dogs with pronounced azotemia (see below).

Hypertension in Blood of Bilaterally Nephrectomized Azotemic Dogs

Dog. No.	Weight	Period after bilateral nephrectomy	Blood pressure	Amount of blood	Incubation	Total yield	Amount injected to test for pressor effect	Rise in blood pressure (direct mean femoral)
	lbs.	days	mm.	cc.	hrs.	cc.	cc.	mm.
10-19†	44	3	125	200	24	90	90	0
10-56†	49	2	125	300	24	130	130	0

† Both dogs had azotemia at the time the sample of blood was withdrawn for detection of a pressor substance. In dog 10-19 the blood urea nitrogen was 192 mg., the creatinine was 9.5 mg., and the CO₂-combining power was 40.1 volumes per 100 cc., while in dog 10-56 the values were blood urea nitrogen 99.8 mg., creatinine 7.2 mg., and CO₂ 43.5 volumes per 100 cc.

tensin present. Part 1 (without hypertensinase action) gave a rise of 60 mm. Hg. Part 2 (with hypertensinase action) gave a rise of 20 mm. Hg.

The same experiment was repeated. About 2 minutes after the intravenous injection of 10 units of hog renin into dog 9-98, 200 cc. of blood was withdrawn. Part 1 (without hypertensinase action) gave a rise of 35 mm. Hg, Part 2 (with hypertensinase action) gave no rise.

In another experiment, the plasma from 200 cc. of blood of a dog with malignant hypertension was treated in the manner described above for the demonstration of hypertensin. Of this plasma, 20 cc. gave a rise of 30 mm. Hg when injected intravenously into a normal

dog. The remaining 60 cc. was divided into three parts. One part was incubated at 40°C. with 10 cc. of normal dog plasma, containing hypertensinase. The hypertensin was completely inactivated in 1 hour. A second part was incubated at 40°C., with a plant hypertensinase preparation, (150 units), derived from wheat bran (8), which does not destroy adrenalin or hydroxytyramine, but does inactivate hypertensin, produced *in vitro*. The hypertensin was completely inactivated in 1 hour. The third part was used as control (not treated) and gave a rise of 30 mm. Hg.

These three tests show that the pressor substance which forms in the systemic blood after the intravenous injection of renin, or after the constriction of the main renal arteries, is destroyed by hypertensinase. Since incubation with plasma containing hypertensinase, or with plant hypertensinase, destroys only hypertensin, and does not affect adrenalin or hydroxytyramine, the pressor substance in the systemic blood of dogs with experimental renal hypertension must be hypertensin or some similar substance.

Chemical Properties of the Pressor Substance in the Systemic Blood of Animals with Experimental Renal Hypertension of the Malignant Type.—Plasma from the systemic blood of dogs with malignant hypertension was used to test for some of the chemical properties which characterize known hypertensin. During the processing of the plasma the substance was subjected to boiling for 10 minutes at pH 4.5, and remained active. Therefore, the substance is heat-stabile and acid-fast. An amount of processed plasma capable of giving a rise of 30 mm. Hg if injected intravenously into an unanesthetized normal dog was boiled for 10 minutes at pH 12.5. After the solution had been cooled, the pH was adjusted to 7.4 with normal HCl. This procedure resulted in complete destruction of the pressor activity. The same amount of processed plasma was dialyzed against cold running tap water for 18 hours. The pressor substance disappeared from the solution in the tubing. The same amount of processed plasma was extracted three successive times with ether, in a separatory funnel, but the pressor activity was not affected by this procedure.

Thus the pressor substance in the circulating systemic blood of dogs with the malignant type of experimental renal hypertension is a non-protein substance, heat-stabile, dialyzable, acid-fast, alkali-labile and ether-insoluble. These qualities are characteristic of hypertensin.

DISCUSSION

Many attempts have been made to demonstrate the existence of a pressor substance in the systemic blood of hypertensive animals:—

Collins and Hoffbauer (9) transfused blood equal to 20 per cent of body weight from a hypertensive dog to a normal dog without obtaining an elevation of blood pressure in the recipient dog.

Katz, Friedman, Rodbard, and Weinstein (10) transfused up to 2500 cc. of blood from a hypertensive dog into a normal dog, by cross-transfusion, without getting a

positive result. Similar experiments, with negative results, have been performed by Houssay and Fasciolo (11). Solandt, Nassim, and Cowan (12) transfused up to 3 liters of blood from hypertensive animals into normal dogs without producing a rise in blood pressure, but they did observe an elevation of blood pressure when such large amounts of blood were transfused into bilaterally nephrectomized animals.

Although I. H. Page (13) was unable to show any pressor effect in 60 cc. of blood from hypertensive dogs, yet later he was able to detect a vasoconstrictor substance in 0.4 cc. of plasma from dogs with experimental renal hypertension, if it was perfused through an isolated rabbit's ear. There is no proof that this effect was due to hypertensin and the work has not been confirmed by others (14).

Heymans and Bouckaert could not demonstrate a pressor substance in up to 20 cc. of blood from hypertensive dogs (15), and others (18, 19) have failed to find hypertensin in the blood of hypertensive patients or animals, or in the blood of animals after an intravenous injection of renin.

The failure of all these attempts to detect the pressor substance in the systemic blood of dogs with experimental renal hypertension can be explained by the inadequate amounts of blood tested and the failure to avoid destruction of the pressor substance by the action of hypertensinase during the tests. Dell'Oro and Braun-Menendez (16) detected renin in both the renal venous blood and in blood from the femoral artery of dogs with experimental renal hypertension that had lasted only a few days. They detected it in as little as 12 cc. of plasma by adding hypertensinogen to enhance the action of the renin and by testing for the hypertensin formed. On this account, their estimate about the amount of renin (100 to 200 units) constantly circulating in the systemic blood of a dog in the earliest stage of experimental renal hypertension is probably too high. The results of our study show that pressor substance is present in small quantity in the circulating blood; therefore, even in the earliest stage, but especially in the later stage of the benign phase of experimental renal hypertension, a large amount of blood must be tested if the hypertensin is to be detected without enhancing the action of renin by the addition of hypertensinogen. This amount corresponds to from one-fifth to one-third of the animal's blood. But even then the pressor substance cannot be demonstrated if the action of the hypertensinase of the plasma is not inhibited. Since 1 cc. of plasma contains almost 1 unit of hypertensinase (17), the 300 to 400 units of hypertensinase can quickly destroy the 1 unit of hypertensin present in this amount of blood, if no precautions are taken to prevent this reaction. If the blood is even slightly hemolyzed, much greater amounts of hypertensinase are available for the destruction of the small amount of hypertensin present in the circulating systemic blood, because red blood corpuscles are rich in hypertensinase, and the detection of hypertensin is therefore likely to fail.

SUMMARY

1. A method has been developed which makes possible the demonstration of a pressor substance in the circulating systemic blood of dogs with experimental renal hypertension.

2. After the intravenous injection of renin into normal dogs, it was possible to detect a pressor substance formed in the systemic blood. After the intravenous injection of 1 unit of renin, as much as 1 unit of the pressor substance was detected in the plasma from 200 cc. of systemic blood.

3. Large amounts of systemic blood pooled from several normal dogs did not contain detectable amounts of pressor substance.

4. In experimental renal hypertension due to unilateral or bilateral constriction of the main renal arteries, a pressor substance was demonstrated in large amounts of systemic blood, corresponding to from one-fifth to one-third of the total blood volume. This was accomplished without the addition of hypertensinogen to enhance the action of the renin in the blood. In an animal weighing about 15 kilos, with benign hypertension up to 3 months' duration, about 3 to 5 units of this pressor substance are probably constantly circulating in the entire systemic blood.

5. The pressor substance was also detected in a relatively small amount of renal vein blood from an ischemic kidney.

6. In the systemic blood of dogs weighing about 15 kilos, with malignant experimental renal hypertension, from 15 to 25 units, or more, of the pressor substance are present in the entire circulating blood.

7. The pressor substance which appears in the systemic blood of dogs with experimental renal hypertension, and of normal dogs after intravenous injection of renin, is destroyed by hypertensinase.

8. The pressor substance obtained from the systemic blood of dogs with experimental renal hypertension has the same physiological and chemical properties as hypertensin produced *in vitro*. It is therefore suggested that the name *hypertensin* be adopted for the pressor substance which causes experimental renal hypertension.

9. In this study the animals in the benign phase of hypertension were almost all in the early stage (3 months or less). Whether the humoral mechanism obtains in animals in the late stage, after years of hypertension, or in any form of human hypertension is being investigated.

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STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

III. SOME FACTORS CONTROLLING THE RATE OF FIBRINOLYSIS*

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The clot which forms when oxalated plasma is recalcified and allowed to stand at 37°C. usually dissolves after a variable period of time. It has been pointed out elsewhere (1) that this fibrinolysis is particularly frequent in patients with chronic hepatic disease. Despite considerable recent interest in the nature of the proteolytic activity of plasma, little attention has been paid to the mechanism by which clotted human blood dissolves. The current report describes certain phenomena which may contribute to an understanding of this mechanism.

In 1893 Dastre (2) observed that canine fibrin was partially digested when it stood in its own serum. Shortly thereafter, Delezenne and Pozerski (3) showed that serum contained a proteolytic enzyme which not only digested fibrin, but gelatin and casein as well. They observed too that treatment of dog serum with chloroform greatly increased its proteolytic activity, and indeed, untreated serum actually inhibited proteolysis. Since then, a large body of evidence has accumulated that the blood contains both a proteolytic enzyme system capable of digesting fibrin, and inhibitors of this enzyme system. The manner in which chloroform acts is not clearly understood, but Delezenne and Pozerski noted that it decreased the inhibitory activity of plasma against proteolytic enzymes. It has been suggested that the proteolytic enzymes present in chloroform-treated plasma globulin are then activated spontaneously, perhaps by autocatalysis (4, 5). As a preliminary hypothesis, then, it might be assumed that the rate of fibrinolysis is determined by the relative concentrations of enzyme and inhibitor.

Rapid fibrinolysis was first described clinically by Goodpasture (6), in patients with cirrhosis of the liver. He noted that the addition of normal plasma to that of a patient with atrophic cirrhosis prevented rapid clot lysis. For this reason, Goodpasture suggested that rapid fibrinolysis in patients with liver disease was due to a decrease in the concentration of an inhibitor of plasma proteolytic enzyme, rather than to an excess of the enzyme itself.

More recently, Macfarlane and Pilling (7) restudied the mechanism of fibrinolysis. A dilute solution of the globulin of plasma precipitated at pH 5.5 promoted clot lysis. Hedin (8) had shown that this fraction exhibited proteolytic activity. Moreover, Macfarlane and Pilling found that the globulin of a patient whose clot lysed rapidly

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was fibrinolytic in a concentration one-eighth that of normal globulin. On the other hand, that fraction of normal plasma not precipitated at pH 5.5 inhibited clot lysis. This latter fraction, obtained from a patient whose clot lysed rapidly, was less inhibitory than that of normal human beings. Macfarlane and Pilling concluded that the spontaneous proteolytic activity observed in their subjects was due to an increase in the concentration of plasma proteolytic enzyme and perhaps to a decrease in the inhibitor of this enzyme as well.

The mechanism of fibrinolysis has been reinvestigated, taking advantage of the apparent parallel between the fibrinolytic and caseinolytic activity of plasma. Data will be presented which indicate that the concentration of the proteolytic enzymes in plasma did not differ between normal persons and those whose plasma clots lysed rapidly. Furthermore the rate of fibrinolysis could not be related to the ability of the patient's plasma to inhibit proteolysis. However, the inhibitory activity of plasma was unstable and decreased during incubation at 37°C. Evidence will be presented which indicates that the speed with which a clot dissolved was related to the deterioration of the unstable inhibitory activity.

Methods and Materials

Venous blood was drawn under aseptic conditions from the antecubital vein of human subjects and transferred to a 50 cc. centrifuge tube containing 0.2 cc. of oxalate mixture for each 5 cc. of blood added (9). The subjects selected were apparently normal adults, personnel or medical students at the Johns Hopkins Hospital, and patients on the medical and surgical wards.

The rate of fibrinolysis was determined on each plasma. The clot lysis time was the interval between the recalcification of plasma and the solution of the resultant clot. Using the necessary technique for sterility, a volume of 0.5 cc. of plasma was pipetted into a Wassermann tube and recalcified with 0.1 cc. of $\frac{1}{20}$ calcium chloride. The number of days which elapsed until the clot dissolved was called the clot lysis time. The sterility of clots which dissolved in 2 days or less was confirmed bacteriologically. A detailed study of the clot lysis time in normal and diseased states has been reported separately (1).

Enzymatic activity was determined by methods previously described (5, 9). The enzyme-substrate mixture was incubated at 37°C., and the degree of proteolysis was determined by assaying the precipitable protein nephelometrically before and after digestion. The substrate used was a 0.3 per cent solution of casein (vitamin-test, Smaco), dissolved with gentle heating in 0.004 N sodium hydroxide. To measure the precipitable protein, 2 cc. of 12.5 per cent hydrochloric acid, and 0.5 cc. of 20 per cent sulfosalicylic acid were added in rapid succession to a 1 cc. aliquot of the solution to be tested. Maximal turbidity appeared in about 5 minutes and was read in a cuvette of 10 mm. internal diameter with a Coleman, Jr., spectrophotometer at a wave length of 420 μ . With the tube, instrument, and lot of casein used, an optical density of 0.225 was equivalent to a concentration of 1.0 mg. of casein per cc. of enzyme-substrate mixture. At a wave length of 420 μ , the degree of turbidity followed the Lambert-Beer law to an optical density of approximately 0.650.

In an earlier publication (5), it was shown that the plasma proteolytic enzymes activated by chloroform or by streptococcal fibrinolysin digested casein in direct proportion to the concentration of the enzyme preparation until approximately 0.7 mg. of casein per cc. remained.

Beyond this point, digestion was not linear. For this reason, it was often convenient to express the activity of proteolytic enzyme preparations in terms of arbitrary digestion units, such that 50 digestion units of enzyme digested 0.50 mg. of casein per cc. of enzyme-substrate mixture in 1 hour.

It should be emphasized that the enzyme and inhibitor fractions prepared in these studies were crude and were not purified products or single substances. A more detailed description of the nomenclature and techniques employed has been given in recent publications (1, 5, 9).

EXPERIMENTAL

The Relationship between the Spontaneous Proteolytic Activity of Plasma Globulin and the Clot Lysis Time

In earlier papers (5, 9), the optimal conditions for the demonstration of proteolytic activity in plasma globulin were described. The spontaneous activity of this preparation was slight. An attempt was made to relate the

TABLE I

The Spontaneous Proteolytic Activity of a Plasma Globulin Preparation and the Clot Lysis Time

Patient	Clot lysis time	Enzymatic activity
	days	mg./cc.*
Du.	1	0.11
Co.	2	0.13
St.	5	0.13
Fr.	6	0.07

* Milligrams of casein digested per cubic centimeter of enzyme-substrate mixture in 16 hours.

rate of fibrinolysis with the spontaneous proteolytic activity of this plasma globulin.

A 2 cc. portion of sterile plasma was diluted with 38 cc. of water in a round bottomed centrifuge tube, and sufficient 1 per cent acetic acid added to bring the pH to 5.2, as measured with a Beckman pH meter. The precipitate which resulted was separated by centrifugation for 15 minutes in an angle centrifuge (rim diameter 10 inches) at 1500 R.P.M., and dissolved in 5 cc. of 0.85 per cent sodium chloride solution buffered to pH 7.35 with M/20 potassium phosphate (buffer). A 2 cc. portion of this globulin solution was then mixed with 2 cc. of 0.3 per cent casein solution, and the amount of casein digested during a 16 hour period of incubation at 37°C. was measured nephelometrically. A few drops of toluol were added as a bacteriostatic agent. The clot lysis time was determined on an aliquot of 0.5 cc. of the same plasma.

The results of one such experiment are recorded in Table I. Plasmas of two patients with Laennec's cirrhosis were compared with those of two normal adults. No correlation was noted between the clot lysis time of these subjects and the spontaneously developing proteolytic activity of a globulin precipitated from the same plasma. This was also true when the globulin was precipitated

from serum rather than plasma. Furthermore, the supernatant solution after the precipitation of globulin at pH 5.2 showed no spontaneous proteolytic activity.

*The Relationship between Chloroform-Activated Plasma Proteolytic Activity
and the Clot Lysis Time*

The major portion of the *in vitro* proteolytic activity of plasma or serum is in an inactive state. Delezenne and Pozerski (3) observed that treatment of serum with chloroform greatly increased its proteolytic activity. It seemed reasonable to suppose that the proteolytic activity of globulin, after treatment with chloroform, was a measure of the total available proteolytic activity. Therefore, the relation was studied between the clot lysis time and proteolytic activity which developed under standard conditions in chloroform-treated globulin.

In previous papers (5, 9), certain conditions were described for the activation of a proteolytic enzyme system in plasma globulin. Briefly, maximal activity was observed when the globulin fraction of plasma precipitated at pH 5.2 was dissolved in buffer, mixed with chloroform, and incubated for 16 to 22 hours at 37°C.

The globulin precipitate of 2 cc. of plasma was prepared in the manner described above, and dissolved in 0.5 cc. of buffer. A half cc. portion of chloroform was then added, the centrifuge tube closed with a rubber stopper, shaken for 10 seconds, and allowed to stand for approximately 18 hours in a water bath at 25°C. The contents of the tube were then transferred to a graduated 15 cc. centrifuge tube, diluted to 5 cc. with buffer, and centrifuged for 10 minutes at 2000 R.P.M. in the angle centrifuge. An aliquot of 2 cc. of the supernatant solution was then mixed with 2 cc. of 0.3 per cent casein, and incubated at 37°C. for 1 hour. The proteolysis of casein was measured by determining the protein concentration of the enzyme-substrate mixture nephelometrically before and after incubation. Enzymatic activity was recorded in terms of arbitrary digestion units per cubic centimeter of plasma.

The chloroform-activated proteolytic activity of the plasma globulin fraction of 83 patients with a wide variety of illnesses including chronic hepatic disease was compared with the clot lysis time determined simultaneously on an aliquot of the same plasma (Fig. 1). No correlation could be demonstrated between the clot lysis time and the chloroform-activated plasma proteolytic activity. That is, the rate of fibrinolysis was not related to the proteolytic activity which appeared in plasma globulin when it was incubated under standard conditions with chloroform.

*The Relationship between Fibrinolysin-Activated Plasma Proteolytic Activity
and the Clot Lysis Time*

It has been demonstrated that the fibrinolytic property of hemolytic streptococcal filtrates was due to the activation of a proteolytic enzyme present in plasma globulin (9-12). The probable identity of the proteolytic enzymes activated by streptococcal filtrates and by chloroform was demonstrated by

these authors. However, the proteolytic enzyme prepared by activating plasma globulin with streptococcal filtrates was always more active than that prepared with chloroform (4, 9). Therefore, the possibility that the concentration of this fibrinolysin-activated enzyme correlated with the clot lysis time was tested.

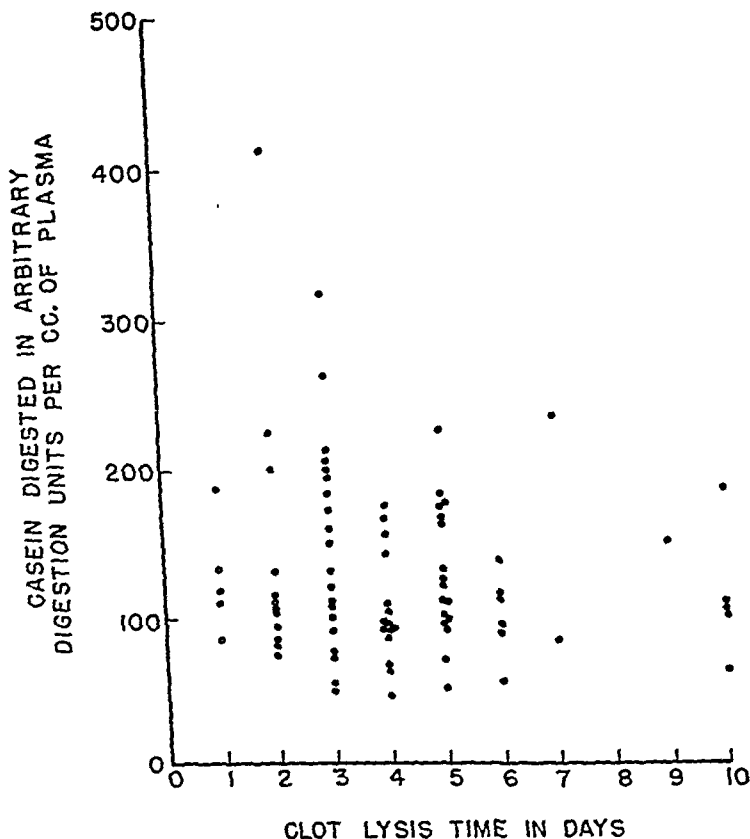


FIG. 1. The relationship between clot lysis time and chloroform-activated plasma proteolytic activity in the globulin fraction of the same plasma in 83 patients.

The globulin precipitate of 2 cc. of plasma was prepared in the manner described above and dissolved in 10 cc. of buffer. An aliquot of 0.5 cc. was diluted with 1.5 cc. of crude streptococcal fibrinolysin, prepared from the H46A strain of group C beta hemolytic streptococcus (9). Briefly, the filtrate of cultures of the streptococcus in trypticase soy broth was used as a crude fibrinolysin. The concentration of fibrinolysin varied somewhat from batch to batch. Each lot was tested to be sure that 1.5 cc. of filtrate was in excess of that amount necessary to activate all the available precursor of proteolytic enzyme present in 0.1 cc. of a test plasma. Approximately 1 minute after the streptococcal fibrinolysin and globulin solutions were mixed, a 2 cc. portion of 0.3 per cent casein solution was added and the protein digested during 1 hour at 37°C. was determined nephelometrically.

The plasmas of 39 patients with various illnesses including chronic hepatic disease were studied. No relationship could be demonstrated between the clot lysis time and the streptococcal fibrinolysin-activated plasma proteolytic activity (Fig. 2).

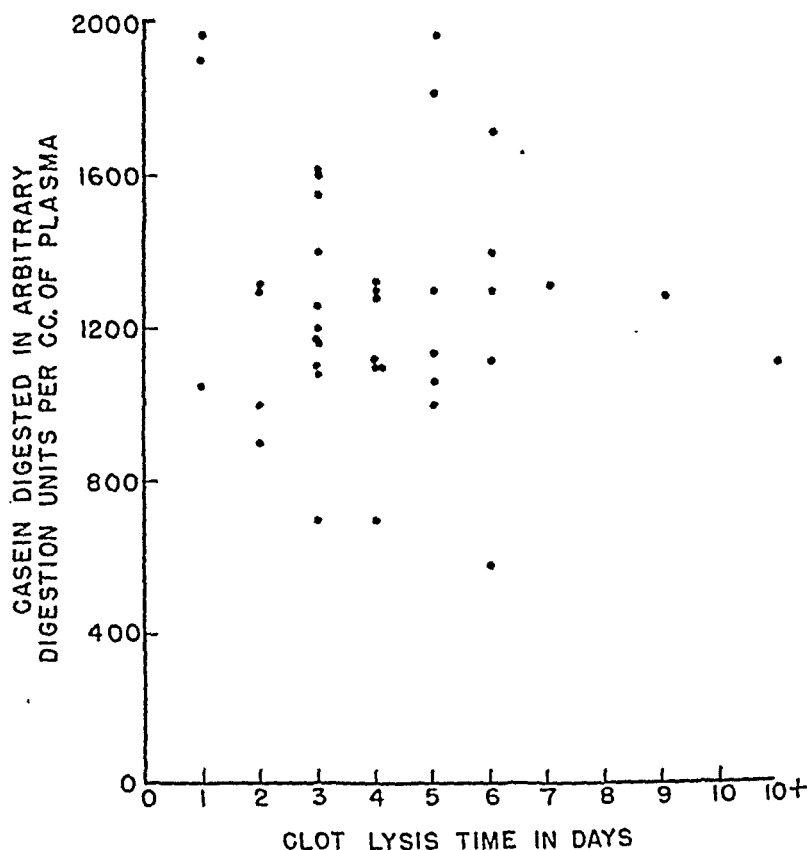


FIG. 2. The relationship between clot lysis time and streptococcal fibrinolysin-activated plasma proteolytic activity in the globulin fraction of the same plasma in 39 patients.

The Relationship between the Ability of Plasma to Inhibit Plasma Proteolytic Enzyme and the Clot Lysis Time

Since rapid fibrinolysis could not be correlated with changes in the available proteolytic activity of plasma, the inhibitory activity of plasma against the proteolytic enzyme was investigated.

To test the inhibitory activity against plasma proteolytic enzyme a measured amount of plasma (or its fractions) was added to enzyme. This enzyme-inhibitor mixture was then mixed with casein and the amount of casein digested

was measured nephelometrically. The enzyme used was a pool of chloroform-activated globulin diluted so that it digested approximately 0.50 mg. of casein per cc. of enzyme-substrate mixture in 2 hours. This concentration was chosen so that the amount of casein digested during the 2 hour period would be directly proportional to the concentration of enzyme, without further correction. Streptococcal fibrinolysin-activated enzyme was not used since any excess fibrinolysin might activate any precursor present in the inhibitor.

A preliminary study was made to determine the optimal length of time to incubate inhibitor and enzyme before the addition of substrate.

In a typical experiment, 1 cc. of plasma was diluted with 29 cc. of buffer. A 5 cc. portion of this diluted plasma was mixed with 10 cc. of chloroform-activated enzyme, and the mixture was then incubated at 37°C. At intervals, aliquots of 2 cc. of the mixture were removed and refrigerated in a salt-water mixture at -10°C. All the tubes were thawed simultaneously and 2 cc. of 0.3 per cent casein added to each. The enzyme-inhibitor-substrate mixtures were then incubated at 37°C. for 2 hours, and the amount of substrate digested determined nephelometrically. This was compared with the proteolytic activity of the same amount of enzyme, mixed with buffer instead of the inhibitory plasma.

The proteolytic activity of the chloroform-activated enzyme was unaffected by incubation at 37°C. for 15 minutes before the addition of substrate. Maximal inhibition of this proteolytic activity was observed when the enzyme and inhibitor were incubated together for 10 minutes before the addition of substrate (Fig. 3).

Quantitative study of the inhibition of plasma proteolytic enzyme requires a knowledge of the relationship between the concentration of inhibitor and the degree of inhibition. Christensen and MacLeod (12) measured the inhibition of chloroform-activated and fibrinolysin-activated plasma proteolytic enzyme by an albumin preparation precipitated from pooled serum and purified by reprecipitation and dialysis two times. They observed that the degree of inhibition was directly proportional to the concentration of albumin. This was in contradistinction to the action of pancreatic trypsin inhibitor, in which the inhibition was proportional to the logarithm of the concentration of inhibitor.

One cc. of fresh plasma or plasma fraction was diluted serially with buffer. One cc. of each of these dilutions of plasma was added to 2 cc. of pooled chloroform-activated enzyme in a Wassermann tube. At the same time, as a control, 1 cc. of buffer was added to 2 cc. of enzyme. The enzyme-inhibitor and enzyme-buffer mixtures were incubated at 37°C. for 10 minutes. A 3 cc. portion of 0.3 per cent casein was then added to each tube, and the amount of protein digested during 2 hours at 37°C. measured nephelometrically.

The data of one such experiment using plasma albumin prepared by precipitation with ammonium sulfate have been plotted in Fig. 4. These indicate that with the enzyme, inhibitor, and substrate employed, the degree of inhibition of chloroform-activated plasma proteolytic enzyme by fresh plasma

albumin was proportional to the logarithm of the concentration of the albumin. In other words, a large change in the concentration of inhibitor led to a relatively small change in the inhibition of the enzyme. The same relationship between the concentration of inhibitor and the degree of inhibition was observed whether the inhibitory substance tested was fresh whole plasma, fresh serum, the albumin precipitated from fresh plasma by ammonium sulfate, or the supernatant "albumin" fraction which remained after the acid precipitation

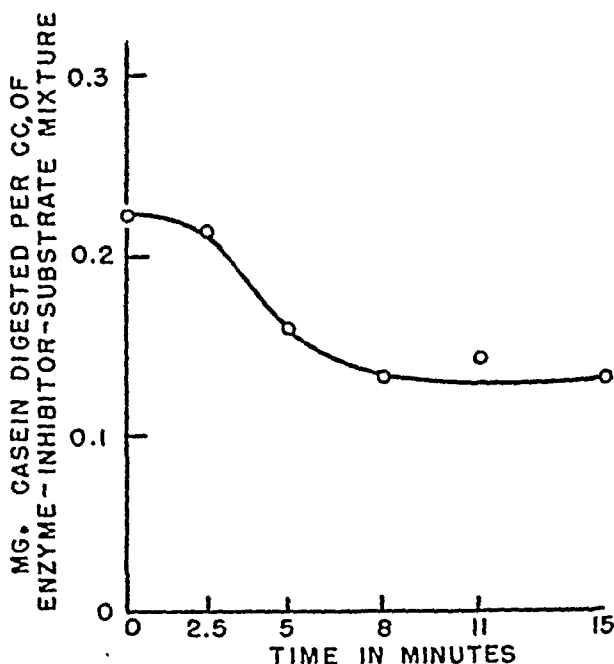


FIG. 3. The relationship between the proteolytic activity of an enzyme-inhibitor-substrate mixture and the duration of incubation of enzyme and inhibitor before the addition of substrate.

of fresh plasma globulin. Moreover, this relationship was found to exist in each of 12 patients tested. Since the method of assay and the substrate employed differed from those employed by Christensen and MacLeod, the differences in result were not necessarily contradictory. The relationship between inhibitor concentration and the degree of inhibition suggests that inhibitor and enzyme react in accordance with the law of mass action.

With this information, the inhibitory activity of the rough albumin fraction of fresh plasma of 33 patients, some of whom had chronic hepatic disease, was tested against chloroform-activated enzyme and compared with the clot lysis time determined on these same specimens of plasma.

A volume of 2 cc. of plasma was diluted with 38 cc. of water and sufficient 1 per cent acetic acid added to bring the pH to 5.2. The resultant precipitate was separated by centrifugation and 8 cc. of the supernatant solution mixed with 2 cc. of buffer. A 1 cc. aliquot of this rough "albumin" fraction was then added to 2 cc. of pooled chloroform-activated plasma proteolytic enzyme. Simultaneously, 1 cc. of buffer was added to 2 cc. of the enzyme as a control. The enzyme pool was such that in the final dilution used it would digest approximately 0.50 mg. of casein per cc. of enzyme-substrate mixture in 2 hours. The enzyme-inhibitor and enzyme-buffer mixtures were incubated for 10 minutes at 37°C. and 3 cc. of 0.3 per cent casein then

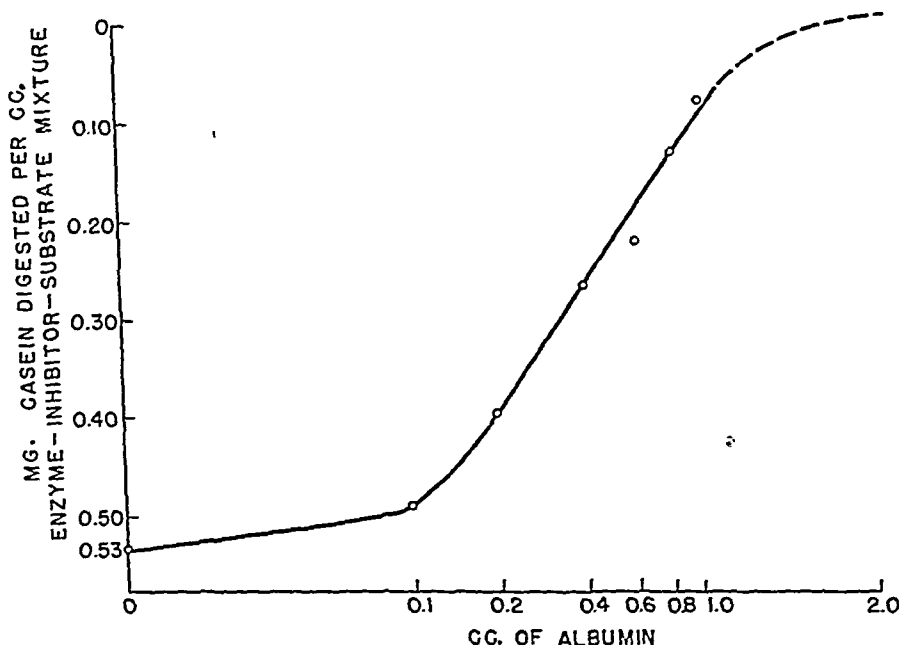


FIG. 4. The relationship between the concentration of plasma albumin and its inhibitory activity against plasma proteolytic enzyme.

added to each tube. The amount of protein digested at 37°C. during a 2 hour period was then determined nephelometrically.

No constant relationship could be demonstrated between the inhibitory activity of the supernatant "albumin" against chloroform-activated plasma proteolytic enzyme and the clot lysis time (Fig. 5). In some experiments, the plasma of patients whose clots lysed rapidly was less inhibitory than that of normal human beings, but this was not a constant finding. Similar studies with fresh whole plasma and serum showed no constant relationship between inhibitory activity and the clot lysis time. That is, the rate of fibrinolysis could not be correlated adequately with the inhibitory activity of fresh plasma, serum, or albumin against plasma proteolytic enzyme.

Changes in the Inhibitory Activity of Serum during the Incubation of a Recalcified Plasma Clot

No definite relationship, then, could be demonstrated between the rate of fibrinolysis of recalcified plasma and the relative available proteolytic activity or inhibitory activity present in plasma. Several alternatives suggested

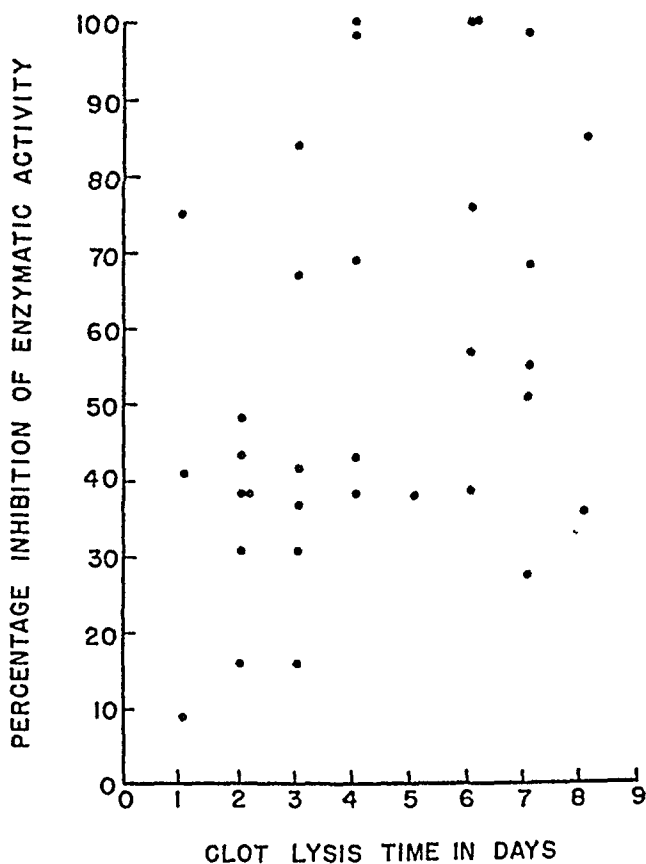


FIG. 5. The relationship between the clot lysis time and the inhibitory activity of fresh plasma albumin against plasma proteolytic enzyme in 33 patients.

themselves. Perhaps caseinolysis was not a measure of fibrinolytic activity; or, on the other hand, mechanisms might have been involved other than those already discussed. The experiments next to be described indicated that the latter hypothesis is at least partially valid.

During the course of these studies it was observed that the plasma of a patient whose clot lysed rapidly was less inhibitory than normal plasma, when the plasmas had been refrigerated at 4°C. for several days before testing. In-

deed, a rough relationship between clot lysis time and the amount of inhibitor in these stored plasmas could be demonstrated. These data intimated that rapid fibrinolysis might be due to a rapid decrease in the inhibitory activity of the serum during the incubation of the clot. The following experiments were designed to test this hypothesis.

Using sterile technique, oxalated plasma from one patient was pipetted in 2 cc. amounts into each of several Wassermann tubes, and recalcified with 0.4 cc. of $M/20$ calcium chloride. The tubes were closed with rubber stoppers, and the clots were then incubated at 37°C . One hour later the serum was expressed from the clot in one of the tubes with a sterile wooden applicator stick. This tube was then centrifuged and the serum stored in a sterile lusteroid tube in a solid carbon dioxide refrigerator at -70°C . Each day the serum of another clot was separated from its fibrin and stored. After the clot had lysed, the serum in other tubes was stored in the same manner from day to day. When the entire series had thus been frozen, all the tubes were thawed simultaneously and 1 cc. of each specimen was then diluted with 19 cc. of buffer. A 1 cc. aliquot of each diluted serum was then added to 2 cc. of chloroform-activated enzyme, and the enzyme-inhibitor mixtures were incubated for 10 minutes. Then an equal volume of 0.3 per cent casein solution was added to each enzyme-inhibitor mixture, and the protein digested during incubation at 37°C . for 2 hours was measured nephelometrically. These studies were performed on the plasmas of several patients, simultaneously, usually comparing a known rapid and slow lysing plasma; the clot lysis times were determined on aliquots of the same plasmas.

The data of several such experiments have been plotted in Fig. 6. These demonstrated that the inhibitory activity of serum decreased rapidly in patients whose clots lysed rapidly, and more slowly in those whose clots lysed slowly. In each experiment the inhibitory activity was approximately the same in each serum when lysis occurred. Once fibrinolysis took place, virtually no further decrease in inhibitory activity was observed, although in no case did all inhibitory activity disappear. In other words, the rate of fibrinolysis could be related directly to the rate of decrease in inhibitory activity of serum when the clot was incubated at 37°C . It will be noted that in these experiments the initial inhibitory activity of the serum of patients whose clots lysed rapidly was usually less than that of patients whose clots lysed slowly. This had not been observed when fresh plasma was tested, and may indicate that some deterioration of inhibitory activity occurred during storage in the carbon dioxide refrigerator.

The nature of the decrease in inhibitory activity was investigated but remained obscure. The effect of the fibrin clot itself or calcium was tested. Oxalated plasma was incubated at 37°C . without recalcification, and 1 cc. portions were removed at intervals and stored at -70°C . These aliquots were thawed simultaneously and their inhibitory activity determined as in the previous experiment. Again the clot lysis time could be correlated with the decrease in inhibitory activity of the plasma. In other experiments, the oxalated plasma was clotted by recalcification. One hour later the serum

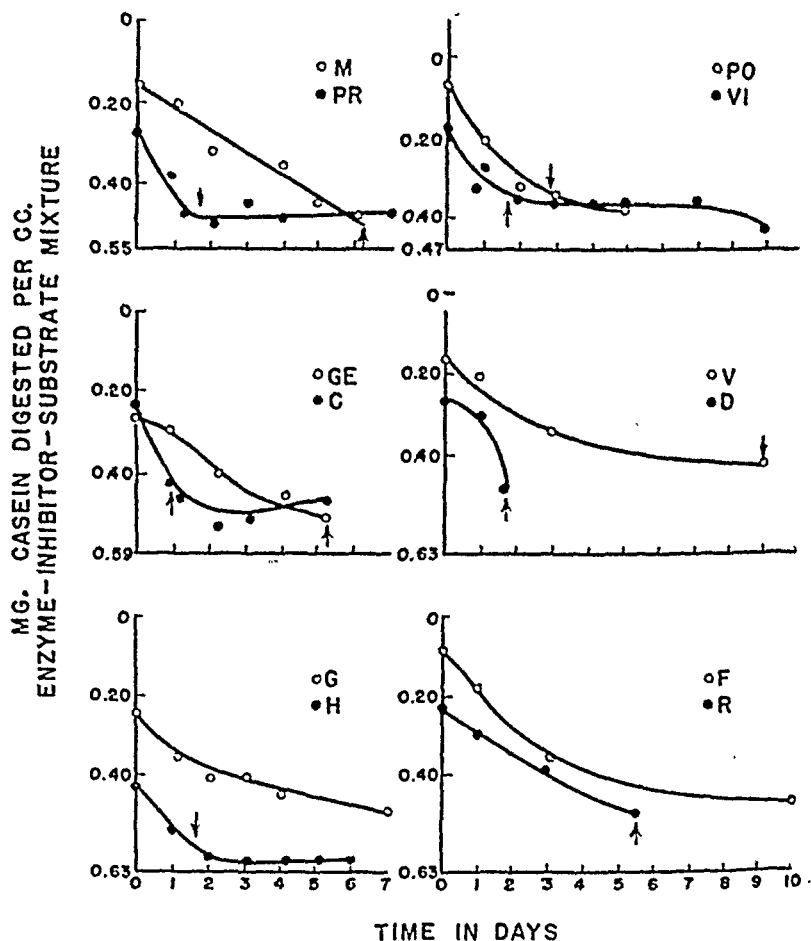


FIG. 6. Changes in the inhibitory activity of serum during its incubation at 37°C., as demonstrated in the sera of 12 patients. Sterile plasma of each patient was pipetted into several Wassermann tubes and recalcified. The clots which formed were then incubated at 37°C. At various intervals serum was expressed from succeeding tubes and frozen. When all the sera had been frozen in this manner, they were thawed simultaneously. The inhibitory activities of the sera of 2 patients were then determined together for comparison. In the figure the inhibitory activity of the serum is recorded as the inverse of the amount of substrate digested. The arrows indicate the time at which the recalcified plasma clot lysed; where no arrow is present, clot lysis did not occur during the period of observation. See text.

was separated from the clot and incubated at 37°C. Aliquots of this serum were removed at intervals and refrigerated at -70°C. The sera were thawed simultaneously, and their inhibitory activities determined. In each case the inhibitory activity of the serum decreased during the period of incubation, and fibrinolysis occurred when the inhibitory activity reached a minimum.

These studies demonstrated that the decrease in inhibitory activity during the incubation of a recalcified plasma clot at 37°C. did not depend upon the presence of calcium or fibrin. Furthermore, the proteolytic activity of the diluted serum or plasma, tested upon the same concentration of casein but with the substitution of buffer for chloroform-activated enzyme, was zero. Therefore, the changes observed could be attributed to a decrease in inhibitory activity rather than a simultaneous increase in the spontaneous plasma proteolytic activity.

The decrease in inhibitory activity was due to a change in the rough albumin fraction. Plasma was incubated at 37°C. and aliquots were refrigerated

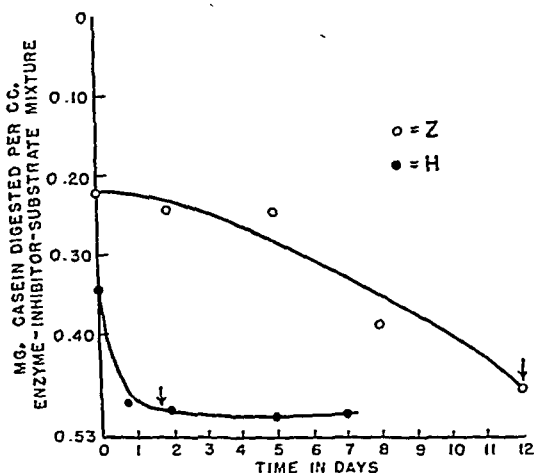


FIG. 7. Changes in the inhibitory activity of the serum albumin during the incubation at 37°C. of sera from patients Z and H. See text.

from time to time as in the earlier experiments. The aliquots were thawed simultaneously, diluted with 19 volumes of water, acidified to pH 5.2, and the globulin precipitate removed by centrifugation. The supernatant "albumin" solutions were diluted with half a volume of buffer, to neutralize the acetic acid. The inhibitory activity of these "albumin" fractions, like that of whole plasma, decreased during the course of incubation at 37°C. (Fig. 7).

In summary, then, the clot lysis time was correlated with the decrease in inhibitory activity against chloroform-activated plasma proteolytic enzyme which occurred during the course of incubation of plasma or serum at 37°C. In those patients whose clots lysed rapidly, the inhibitory activity of the plasma, serum, or albumin reached a minimum more rapidly than normally. After clot lysis had occurred, there was little or no further decrease in the amount of inhibitor present, although inhibitory activity was not completely abolished. The phenomenon was unrelated to the presence of calcium or fibrin.

DISCUSSION

The experiments which have been described hint at possible explanations for the phenomenon of rapid fibrinolysis occasionally observed in human plasma. The clot lysis time could not be correlated with the total available proteolytic activity which resides in plasma globulin, at least as measured by the several techniques used. On the other hand, no relationship was demonstrated between the clot lysis time and the ability of fresh plasma to inhibit plasma proteolytic enzyme. However, when recalcified plasma was incubated at 37°C., its inhibitory activity against plasma proteolytic enzyme gradually decreased until a relatively stationary minimum was reached. This occurred at approximately the time that the clot lysed. In any given experiment, the minimal level of activity of several plasmas was approximately the same, but the rate at which this minimal level was reached varied with the rate of fibrinolysis.

No attempt was made to correlate rapid fibrinolysis with the amount of fibrinogen in these plasmas. However, Geill (14) and others have demonstrated that the concentration of fibrinogen in the plasma of patients with all but the most catastrophic liver disease is either normal or slightly elevated. Consequently, it seems unlikely that a decrease in available substrate is a common concomitant of rapid fibrinolysis.

The nature of the decrease in the inhibitory activity of plasma is not clear. This is due in part to a dearth of information concerning the chemical composition of the inhibitory substance or substances. It was apparently not dependent upon the presence of calcium or fibrin. A similar phenomenon was described by Jobling and Petersen (13) in 1914, but they did not relate it to clot lysis. These authors reported that the trypsin-inhibiting activity of serum decreased 5 to 10 per cent during the first few days of incubation and then remained constant indefinitely.

Perhaps the inhibitory activity of plasma depends upon a mixture of labile and stable components. The labile component deteriorates during the incubation of the clot at 37°C. Thus far, attempts to define the nature of this deterioration have not been illuminating. Certain preliminary observations suggest that oxidation may be one of the ways that labile inhibitor is destroyed. It was observed that clot lysis was always more rapid in Wässermann tubes which were plugged with cotton compared with those closed with rubber stoppers; but ready access to oxygen was not the only variable in such experiments. Furthermore, mild oxidants were shown to speed clot lysis, but the data are sufficiently equivocal not to permit any definite conclusions. It is of interest that the inhibitory activity of plasma was recently found by Grob (15) to be diminished in the presence of oxidants. Moreover, he observed that reconstituted proteins, prepared from the purified plasma protein fractions of Cohn and his associates, had only a small fraction of the inhibitory

activity of fresh serum or plasma against plasma proteolytic enzyme, when tested by methods similar to those reported here. In the present study, similar observations were made upon individual protein fractions.¹ This suggests that the labile inhibitor may not be precipitated with the proteins or may have been destroyed during the fractionation process.

Finally, several alternative hypotheses might explain the mechanism of deterioration of the labile inhibitor of plasma proteolytic enzyme. Conceivably, there may be an enzyme in plasma which destroys the labile inhibitor, and such an enzyme may be present in excess in patients with short clot lysis times. Or the labile inhibitor of patients with short clot lysis times may be qualitatively more susceptible to deterioration than that of normal human beings. Or some substance or substances present in normal plasma may be capable of slowing the deterioration of labile inhibitor, and these substances may be lacking in the patient with rapid fibrinolysis. These various hypotheses are now the subjects of further investigation.

SUMMARY

Methods are described for the assay of chloroform-activated and fibrinolysin-activated plasma proteolytic enzyme, and for the determination of the inhibitory activity of plasma or other substances against chloroform-activated enzyme.

The inhibitory activities of plasma, serum, and crude plasma albumin against chloroform-activated plasma proteolytic enzyme were proportional to the logarithm of the concentration of the inhibitory substance tested. This suggests that enzyme and inhibitor react in accordance with the law of mass action.

The rate of fibrinolysis of recalcified plasma clots could not be related to the total proteolytic activity available in the plasma, nor to the inhibitory activity of fresh plasma or serum against plasma proteolytic enzyme.

During the incubation of a recalcified plasma clot at 37°C., the inhibitory activity of its serum against plasma proteolytic enzyme decreased until a minimal stationary level was reached. The clot lysis time could be correlated directly with the time which elapsed until this minimal level was reached.

It is a pleasure to acknowledge the advice and encouragement of Dr. George S. Mirick.

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STUDIES ON ACUTE DISSEMINATED ENCEPHALOMYELITIS PRODUCED EXPERIMENTALLY IN RHESUS MONKEYS. III*

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PLATE 22

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Earlier studies from this laboratory (1, 2) and by Morgan (3) have independently established that administration of homologous or heterologous brain tissue in an emulsion with paraffin oil, aquaphor, and killed tubercle bacilli as adjuvants (4) would induce an acute disseminated encephalomyelitis in *rhesus* monkeys more rapidly than had previously been accomplished without adjuvants (5, 6) and these findings have been confirmed by Ferraro and Cazzullo (7). Freund, Stern, and Pisani (8) and Morrison (9) have produced a similar disease in guinea pigs and in rabbits, but the pathological changes in these species were much less striking, notably with respect to the degree of demyelination. In the studies with rabbits (9, 10), however, there were sometimes animals which showed disease symptoms, but failed to show any pathological changes at necropsy, and conversely there were animals which remained completely free of symptoms but in which pathological changes were found; moreover in both guinea pigs (8) and rabbits (9) the total incidence of the disease was lower than in monkeys. Since in the monkey, there was almost always complete agreement between the symptoms and the pathological findings, except in those few animals in which the symptoms could be demonstrated by necropsy to have an entirely unrelated cause, or in an occasional animal which showed very few pathological changes without symptoms having been noted, it was decided to continue the study of the etiology of this disease in the monkey.

The work has been based on the hypothesis that the disease is the result of an immunological response to the injected brain tissue and that the lesions result from the interaction between the brain tissue of the host and antibrain antibody formed to the injected material (1-3) or, alternately, that the injections induced sensitization of the tuberculin type (8), which would be associated with cellular rather than with humoral antibodies. The present report summarizes data obtained on the distribution of the inciting antigen in the brains of various animal species, on the appearance of this antigen in the cerebrum and spinal cord of rabbits of varying ages after birth in relation to the degree of myelina-

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tion, and on the stability of the antigen in brain tissue. Data are also given concerning attempts to produce the disease by passive transfer with the serum as well as with cell exudates and suspensions of spleen and lymph nodes of monkeys with disseminated encephalomyelitis, and with the serum of rabbits immunized with emulsions of brain tissue with adjuvants. The mechanism of the rôle of tubercle bacilli in the induction of the disease in monkeys is also considered.

EXPERIMENTAL

Emulsions were prepared as described in (1) and were standardized on the basis of wet weight as follows: 7 gm. of brain or spinal cord was homogenized in a Waring blender in 10 ml. saline containing 1 per cent phenol, 10 ml. of molten aquaphor, and 20 ml. of paraffin oil containing 25 mg. of heat-killed tubercle bacilli (strain H37RV). The final emulsion was heated at 60°C. for 45 minutes (1). Each milliliter of emulsion contained 175 mg. of original tissue. When larger amounts of tissue were used, the volumes of all constituents were increased proportionately. In experiments involving extraction with alcohol or acetone, a given weight of tissue was extracted, the extract evaporated to dryness at room temperature and the residue dried and emulsions of each prepared based on the original weight of tissue extracted.

Injection of Animals.—Each monkey received an initial course of three 1 ml. intramuscular injections. Injections were spaced a week apart. The animals were then observed daily for 6 weeks to 2 months. Monkeys which had shown no symptoms were given a second course of three 1 ml. injections and observed for an additional 3 months after which all animals were sacrificed. Monkeys which showed symptoms were observed until it appeared unlikely that they would survive another day. They were then sacrificed by exsanguination and a complete necropsy performed (1, 2), including gross and microscopic examination. Animals listed in the table as positive showed symptoms as well as lesions typical of acute disseminated encephalomyelitis at necropsy. Animals listed as negative showed neither symptoms nor pathological findings of disseminated encephalomyelitis. In a few instances symptoms, usually generalized weakness, were recognized by necropsy to be the result of unrelated disease.

RESULTS

The capacity of various materials, when incorporated in emulsions with aquaphor, paraffin oil, and killed tubercle bacilli, to induce acute disseminated encephalomyelitis in *rhesus* monkeys is shown in Table I. Although chicken, monkey, and human brain were effective in inducing this disease, frog and fish brain gave negative results. With both human and monkey brain emulsions, negative results were obtained if the tubercle bacilli were omitted. Negative results were also obtained in groups of monkeys in which human or monkey brain emulsions containing aquaphor and paraffin oil but lacking tubercle bacilli were injected into one side of a group of animals and an emulsion of aquaphor, paraffin oil, and tubercle bacilli but containing no brain tissue was injected into the opposite side.

Data on the stability of the antigen in brain tissue are also included in Table I. It may be seen that the capacity of brain tissue to induce the lesions was not significantly affected by exposure to ultrasonic vibration at 600 kc. for two 10

TABLE I

Polency of Various Emulsions with Adjuvants in Producing Acute Disseminated Encephalomyelitis in Rhesus Monkeys

Material injected*	No. of monkeys used	First course No. of monkeys positive	Second course No. of additional monkeys positive	No. of monkeys positive No. of monkeys used
Chicken brain.....	3	2		2/3
Frog brain.....	4	0	0	0/4
Fish (carp) brain.....	3	0	0‡	0/3
Human brain.....	5	2	2	4/5
Human brain (without tubercle bacilli).....	3	0	0	0/3
Human brain, paraffin oil, aquaphor, without tubercle bacilli in right side; paraffin oil, tubercle bacilli, aquaphor in left side.....	4	0	0§	0/4
Human brain (ultrasonically treated).....	3	2‡	1	3/3
Human brain (alcoholic extract).....	3	0	0	0/3
Human brain (residue from alcoholic extraction).....	3	0	0	0/3
Monkey brain.....	5	4	0	4/5
Monkey brain (without tubercle bacilli).....	3	0	0	0/3
Monkey brain, paraffin oil, aquaphor, without tubercle bacilli in right side; paraffin oil, tubercle bacilli, and aquaphor in left side.....	4	0	0	0/4
Monkey brain (autolyzed**).....	3	1	0	1/3
Monkey brain (boiled‡‡).....	3	2	0	2/3
Monkey brain formalinized.....	4	4		4/4
Monkey brain (acetone extract).....	3	0	0	0/3
Monkey brain (residue from acetone extract).....	3	0	1	1/3
Monkey brain (alcohol extract).....	3	0	0	0/3
Monkey brain (residue from alcoholic extract).....	3	0	0	0/3
Monkey peripheral nerve§§.....	7	0	0	0/7

* All emulsions were from adult animals and were made up with aquaphor, paraffin oil, and tubercle bacilli except where otherwise noted.

‡ Animals received only two injections in second course.

§ Three animals did not receive a second course.

|| Saline suspension of brain exposed to ultrasonic vibration at 600 kc. for two 10 minute intervals.

‡ One of these animals died, was accidentally disposed of without being necropsied. The animal showed ataxia, ptosis of both eyelids, rotation of head and weakness of right hind leg, and was considered positive.

** The brain was homogenized in saline without phenol, 1 ml. of toluene was added, and the material allowed to stand at room temperature for 22 days, before adding adjuvants.

‡‡ Brain homogenate in saline heated in boiling water bath for 1 hour.

§§ One animal died before second course was begun, one animal received only one injection of second course, and three animals received only two injections of second course.

|| || This animal showed very few lesions at necropsy and no symptoms were observed.

minute intervals, by heating in saline suspension in a boiling water bath for 1 hour, or by fixation in 10 per cent formalin as employed for routine pathological study. After extraction of brain tissue with alcohol or with acetone neither the extract nor the extracted residues showed any capacity to induce encephalomyelitis except for very mild lesions without symptoms discovered on histopathological examination in one animal injected with the acetone residue. One of three animals injected with an emulsion of autolyzed monkey brain developed encephalomyelitis; data are not yet sufficient to establish whether autolysis resulted in any loss of potency. In confirmation of the findings of

TABLE II

Development in Rabbit Brain and Spinal Cord of Antigen Inducing Acute Disseminated Encephalomyelitis

Material injected	No. of monkeys used	First course No. of monkeys positive	Second course No. of additional monkeys positive	No. of monkeys positive No. of monkeys used
Cerebrum from 3 mos. old rabbit....	6	2	1	3/6
" " 2 " " "	7	3	1	4/7
" " 1 " " "	6	6	0	6/6
" " 12 day " " "	7	1	1	2/7
" " 6 " " "	7	0	0	0/7
" " 3 " " "	7	0	0	0/7
" " 1 " " "	3	0	0	0/3
Spinal cord from 12 day rabbit.....	4	3	0	3/4
" " " 6 " "	4	3	0	3/4
" " " 3 " "	4	0	3	3/4

All materials incorporated into emulsions with adjuvants.

Morgan (3), negative results were obtained by inoculation of emulsions of monkey peripheral nerve.

Since it previously had been demonstrated (1) that the antigen was absent from fetal rabbit brain, its appearance during early life was investigated. The results of injection of emulsions with adjuvants of the cerebrums and spinal cords of groups of rabbits of varying ages are shown in Table II. It is evident that the substance inducing encephalomyelitis did not appear in the rabbit cerebrum until the animals were 12 days of age whereas it was present in the spinal cord of 3 day old rabbits.

Table III summarizes attempts to induce acute disseminated encephalomyelitis passively with the serum of monkeys suffering from the disease and with the serum of rabbits immunized with emulsions of brain tissue with adjuvants following the same schedule used to induce the disease in monkeys.

Completely negative results were obtained even in three monkeys which had received intravenous injections of 200 ml. of serum from seven to ten monkeys with encephalomyelitis, in two animals after giving 21 ml. of monkey serum

TABLE III

Attempts to Produce Acute Disseminated Encephalomyelitis by Passive Transfer with Serum

Material used.	Monkey No.	No. of injections	No. of serum donors	Total volume injected	Time between 1st and last injections	Day of death	Symptoms and pathological findings
				ml.	days		
Injections intravenously							
Serum from monkeys with acute disseminated encephalomyelitis	2-3	8	3	40	54	86(S*)	Negative
	2-4	7	3	35	47	82(S)	"
	3-6	5	3	25	25	128(S)	"
	3-7	5	3	25	25	128(S)	"
	1-67	11	9	200	14	94(S)	"
	1-76	10	10	200	12	94(S)	"
	1-19	11	7	200	20	39	"
Injections intracisternally							
	9-1	21	4	21	130	140	Negative
	9-2	21	2	21	130	211(S)	"
Injections intravenously							
Serum from rabbits immunized with monkey brain tissue containing adjuvants†	8-6	13	10	68	55	57	Negative
	8-7	13	4	68	55	58	Neurological symptoms shown at autopsy to be of serum sickness type
	1-08	30	15	147	58	151(S)	Negative
	1-09	32	13	160	62	96	"
	1-68	20	10	200	43	94(S)	"

* Sacrificed.

† Rabbits received schedules of injections similar to those used to induce encephalomyelitis in monkeys, and were bled 10 days and 1 month after each course.

intracisternally, and in five monkeys given 68 to 200 ml. of serum from rabbits immunized with emulsions of brain tissue.

Data on unsuccessful attempts to effect passive transfer of acute disseminated encephalomyelitis by the technic of cellular transfer which has been shown by Chase (11) to be effective in passive transfer of the tuberculin type of reaction are presented in Table IV. Peritoneal exudates produced as described by

Chase by intraperitoneal injection of 40 ml. of paraffin oil into monkeys suffering from encephalomyelitis and suspensions in Tyrode's solution containing monkey serum of spleen and lymphoid tissue obtained under aseptic precautions were employed. Negative results were obtained in all instances even

TABLE IV

Attempts to Produce Acute Disseminated Encephalomyelitis by Passive Transfer of Cells from Abdominal Exudates and from Splenic and Lymphoid Tissue

No. of recipient	Source of cell suspension	No. of injections	Days between 1st and last injection	No. of donor monkeys	Total volume of cell suspension injected ml.	Day of death	Symptoms and pathological findings
1-39	Abdominal exudate	9 ip* 8 im*	25 25	2	60 59	153(S)	No symptoms†
1-40	Abdominal exudate, spleen and lymph node	24 ip 21 im	147 34	4	218 182	204(S)	Negative for encephalomyelitis
1-44	Abdominal exudate, spleen and lymph node	3 ip 3 im	17 17	2	21 21	127(S)	" "
1-45	Abdominal exudate, spleen and lymph node	17 ip 14 im	139 26	4	168 131	222(S)	" "
1-74	Spleen and lymph node	7 ip	69	7	101	122(S)	" "
1-75	Spleen and lymph node	7 ip	69	7	101	122(S)	" "
2-23	Abdominal exudate, spleen and lymph node	2 ip	4	2	17	17(S)	" "

* ip = intraperitoneal, im = intramuscular.

† Not autopsied.

in two recipient monkeys each of which had received one-half of the spleen and lymph node suspensions of seven donor monkeys with encephalomyelitis.

The inoculation sites of all animals which received injections of brain emulsions with and without tubercle bacilli were sectioned and examined microscopically. When killed tubercle bacilli were omitted, the reaction at the inoculation site in striated muscle and subcutaneous tissue was quite mild

(Fig. 1 *a*). Narrow trabeculae of connective tissue about small lacunae containing amorphous material marked the area in which absorption of the brain emulsion occurred and this corresponded to fine fibrous veining in pale, poorly outlined areas of muscle in the gross specimen which were frequently quite inconspicuous. The connective tissue trabeculae contained small clusters of lymphocytes. This was in sharp contrast to the conspicuous lesions produced by the injection of an emulsion containing both brain and adjuvant materials (Fig. 1 *b*) which resulted in striking local lesions in the muscle and subcutaneous tissue. Masses of meaty, compact greyish white or white firm tissue containing varying sized pockets of yellowish or cream colored exudate were seen. Histologically these proved to be masses of epithelioid cells, usually poor in giant cells although occasionally containing moderate numbers of them. Spaces of various size in the granulation tissue contained amorphous material and polymorphonuclear leucocytes tended to cluster near these. Lymphocytes were usually abundant, both diffusely spread and in clusters. As these lesions grew older fibrosis occurred and plasma cells appeared while polymorphonuclear leucocytes diminished and disappeared. The adjuvant material without the brain tissue produced a lesion much like the complete mixture (Fig. 1 *c*).

DISCUSSION

The results obtained provide some additional information on the nature of the substance in brain tissue which, when injected with adjuvants, induces the appearance of acute disseminated encephalomyelitis in the monkey. The antigen is apparently organ-specific since brain from a species even as remote in zoological relationship as the chicken will induce the disease; however, brains from more distantly related animals like the frog and fish give negative results. The antigen is fairly stable since it withstood fixation in formalin, heating in a boiling water bath, and treatment with ultrasound. It is, however, destroyed by alcohol extraction, both the extract and the residue being inactive. An acetone extract was completely inactive and the residue showed slight residual activity. The finding in Table II that the antigen is present in the spinal cord of 3 day old rabbits, but does not appear in the cerebrum of rabbits until the 12th day of life indicates a close parallelism with the laying down of myelin which proceeds in the same sequence and suggests that myelin may be the antigen involved. This is of interest in relation to the findings of Schwentker and Rivers (10) that the organ-specific antigen in autolyzed brain as measured by complement fixation paralleled its myelin content. A final decision as to the nature of the antigen, however, must await its isolation from brain tissue in purified form and a study of its chemical and physical properties. In confirmation of the report by Morgan (3), monkey peripheral nerve emulsions were found not to induce the disease.

The unsuccessful attempts to effect passive transfer of the acute disseminated

encephalomyelitis either with serum or with cell exudates from affected monkeys or with the serum of rabbits injected with brain emulsions with adjuvants leave a serious gap in the chain of evidence supporting the hypothesis (1) that an antibody to the injected brain tissue reacts with the nervous system of the animal to produce the disease, since no positive evidence for such an antibody has yet been obtained. As previously noted (1) this does not necessarily contradict or weaken this hypothesis since the hypothetical antibody would be continuously removed from the circulation by the tissues of the central nervous system of the donor monkey and the amount present even in the 200 ml. of serum injected passively might be inadequate to produce the disease. Kolb and Bolton (12) were unable to produce lesions in the central nervous system of rats with rabbit antisera to rat and cat brain and Hurst (13) refers to the negative results of Hurst and Atkinson on injection of pigs with antiserum to pig brain produced in sheep and rabbits.

The experimental findings in Table I and the histological studies of the inoculation sites provide some indication of the mechanism of action of the tubercle bacilli and of the induction of the disease. As seen in Table I not only are the tubercle bacilli necessary for the development of acute disseminated encephalomyelitis with the injection schedule employed, but, indeed, they must be present at the site at which the brain emulsion is injected, since injection of emulsions of brain without tubercle bacilli into one side and of tubercle bacilli without brain into the other side yielded negative results. Histologically, the sites containing tubercle bacilli showed much more pronounced local reactions with large masses of epithelioid cells which were absent in sites without tubercle bacilli. Were the enhancing effect merely a systemic response to the injection of tubercle bacilli, positive results should have been obtained when emulsions with tubercle bacilli but without brain and of brain without tubercle bacilli were injected at different sites. It is very tempting to infer that the function of the tubercle bacilli in producing encephalomyelitis more rapidly than can be accomplished otherwise is to bring these epithelioid cells in close proximity to the antigen and that the formation of antibody to the brain antigen takes place locally at the inoculation site (for evidence on the local formation of antibodies compare references 14 and 15).

SUMMARY

The factor in brain tissue which induces acute disseminated encephalomyelitis, when injected into *rhesus* monkeys as an emulsion with adjuvants, has been found in human, monkey, rabbit, and chicken brain but is absent from frog and fish brain. It is unaffected by fixation of the brain in formalin, by boiling, and by treatment with ultrasound. It is present in the spinal cord of 3 day old rabbits but does not appear in the rabbit cerebrum until about the 12th day of life; in this respect it parallels the laying down of myelin. Attempts

to produce the encephalomyelitis passively with large quantities of serum or of cell exudates, and suspensions of cells from spleen and lymph node from monkeys with encephalomyelitis, were unsuccessful.

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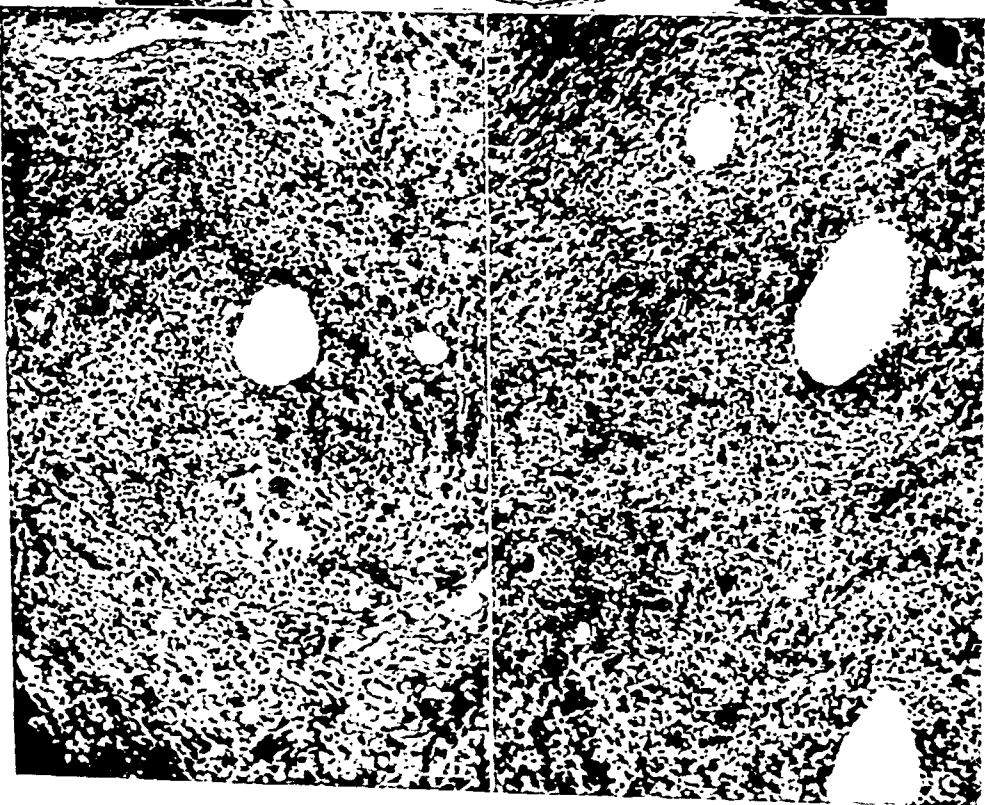
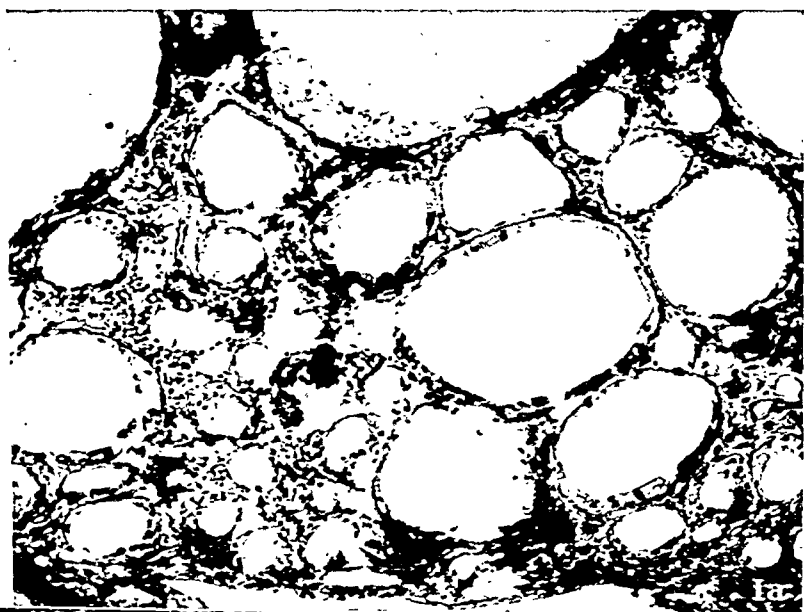
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EXPLANATION OF PLATE 22

FIG. 1 *a*. Monkey 9-7. Inoculation site in right thigh. Mild infiltration by lymphocytes and occasional epithelioid cells about spaces in which inoculum lay. Inoculum composed of monkey brain, aquaphor, and paraffin oil without killed tubercle bacilli. Hematoxylin-eosin stain. $\times 75$.

FIG. 1 *b*. Monkey 9-9. Inoculation site in right thigh. Abundant granulation tissue containing a great many epithelioid cells, numerous lymphocytes, and occasional multinucleated giant cells. Inoculum composed of monkey brain, aquaphor, paraffin oil, and killed tubercle bacilli. Hematoxylin-eosin stain. $\times 75$.

FIG. 1 *c*. Monkey 1-54. Inoculation site in left thigh. Granulation tissue like that in Fig. 1 *b*. Inoculum composed of aquaphor, paraffin oil, and killed tubercle bacilli but lacking monkey brain. Hematoxylin-eosin stain. $\times 75$.



(Kabat *et al.*: Acute disseminated encephalomyelitis. III)

KINETIC STUDIES ON IMMUNE HEMOLYSIS*

I. A METHOD†

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One approach toward a better understanding of the mechanism of immune hemolysis is the study of its kinetics. While a number of investigations along these lines have been undertaken (1-11), most of the data lack the precision and scope required for quantitative kinetic analysis. Only a few studies have included measurements of the rate of the reaction over its entire course, and most of these have dealt solely with the effect of varying the concentration of complement (C'). Other factors, such as the concentration of antibody or of red cells, and the effect of pH, temperature, and salt concentration, etc., have been evaluated only by end point measurements, such as the usual dilution titers, or the determination of the time required for complete lysis, as, for example, in references 9 and 10. Furthermore, in many of the early experiments (1-4) no clear distinction was made between the effect of hemolytic antibody and of C' , since these agents were not introduced as separate entities.

The need for a better understanding of the kinetics of immune hemolysis became acutely apparent to us as a result of unsuccessful attempts to develop a quantitative method for the titration of hemolytic antibody, analogous to that employed in the estimation of C' (12). Measurements of reaction rates showed the difficulties to be due to the fact that the kinetics of hemolysis in systems containing an excess of C' and limited amounts of antibody are strikingly different from those of systems in which antibody is present in excess and C' is limited in quantity.

The present technique of measuring the rate of hemolysis by antibody and C' is based on the spectrophotometric method of C' titration (12), as well as on the elucidation of the rôle of Mg^{++} and Ca^{++} in immune hemolysis (13). The capacity of citrate ion to inhibit hemolysis by binding Mg^{++} and Ca^{++} has been utilized as a means of stopping the hemolytic process at any desired time, eliminating the need for immediate and rapid centrifugation of samples with-

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drawn for analysis as in reference 9. In this manner considerable improvement in the timing of analyses as well as in ease and convenience of experimentation has been achieved.

The experiments described and discussed below have been assembled to illustrate the details of procedure as well as to outline some of the initial results of kinetic analysis. Further investigations are in progress and will be dealt with in subsequent communications.

EXPERIMENTAL

Materials and Methods.—Sheep blood was preserved aseptically in modified Alsever's solution (14), and standardized suspensions of washed sheep erythrocytes were prepared as in reference 12 except that the concentration of erythrocytes was adjusted so that a 1.0 ml. portion of cell suspension mixed with 14.0 ml. of a 0.1 per cent solution of anhydrous sodium carbonate¹ yielded a lysate of optical density 0.34 read in the Beckman quartz spectrophotometer at a wave length of 541 $m\mu$ ² in a cuvette of 10 mm. light path and with distilled water as reference standard. Cell suspensions so prepared from the blood of healthy, adult sheep³ contained 5×10^8 erythrocytes per ml.

Since Ca^{++} and Mg^{++} are essential constituents of the hemolytic system (13) these ions were supplied in optimal concentrations by means of a veronal-bicarbonate buffer⁴ containing 0.00015 M $CaCl_2$ and 0.0005 M $MgCl_2$. During the early stages of the work this served as wash fluid as well as diluent for the erythrocytes and the guinea pig C'. Later, the red cells were washed with and suspended in buffer containing 0.1 per cent crystalline bovine serum albumin (SA)⁵ in addition to the other components, since less spontaneous lysis occurred under these conditions.

Hemolytic antisera were prepared in rabbits by intravenous injection of 11 ml. of a 10 per cent suspension of washed sheep erythrocytes administered in 6 doses over a period of 2 weeks. After 10 days' rest the animals were bled 40 ml. by cardiac puncture. A second course of 6 injections was then given during the following 2 weeks with a total of 15 ml. of 10

¹ Sodium carbonate solution, unlike distilled water, yields optically clear lysates.

² The red color of the lysate, due to oxyhemoglobin, is characterized by light adsorption maxima at wave lengths of 541 and 576 $m\mu$, respectively, and an absorption minimum at 559 $m\mu$. During the prolonged periods of incubation used in the present experiments a small part of the oxyhemoglobin is converted to methemoglobin. At 576 $m\mu$ the optical density of methemoglobin at pH 7.4 equals 28 per cent of that of oxyhemoglobin, while at 541 $m\mu$ it is 43 per cent. Therefore the analytical error due to conversion of oxy- into methemoglobin is smaller if optical densities are determined at 541 $m\mu$. In some of the later experiments the extent of methemoglobin formation was estimated spectrophotometrically (15) and the resulting error in the measurement of hemolysis was found to be 1 per cent after 1½ hours, and about 4 per cent after 8 hours of incubation at 37°C.

³ The blood was obtained from a colony of 4 sheep, and each animal supplied 400 ml. of blood approximately every 8 to 12 weeks. Bloods were not pooled.

⁴ 83.8 gm. NaCl, 2.52 gm. $NaHCO_3$, 3.00 gm. sodium 5,5-diethyl barbiturate, 4.60 gm., 5,5-diethyl barbituric acid, 1.0 gm. $MgCl_2 \cdot 6H_2O$, 0.2 gm. $CaCl_2 \cdot 2H_2O$. Dissolve the acid in 500 ml. hot water, add to the solution of the other components, cool, and make up to 2000 ml. with water. Each day dilute accurately 1 part up to 5 with water. The pH of the diluted buffer should be 7.3 to 7.4.

⁵ Armour and Company, Chicago, Illinois.

per cent erythrocyte suspension per animal. Thirteen days after the last injection a second bleeding was taken. Antiserum pool A contained the second bleedings from rabbits 29, 30, 31, and 32. Antiserum B was pooled from the first bleedings of rabbits 29, 30, and 32. The bulk of each pool was stored at -20°C . and a small portion, accurately diluted 1/10 or 1/20 with saline, was kept in the refrigerator. From this stock, further dilutions were made each day with veronal-bicarbonate buffer⁴ containing 0.008 per cent SA (subsequently referred to as SA-buffer). In later experiments the concentration of albumin in the diluent was raised to 0.1 per cent. In agreement with similar observations in reference 16, preliminary experiments indicated loss of antibody activity due to adsorption on glass when plain buffer was used as diluent, but not when SA-buffer was used.

For C', pools of 30 to 60 ml. of serum from 10 to 20 guinea pigs were absorbed 4 to 6 times for $\frac{1}{2}$ hour in the cold with washed, packed sheep erythrocytes (1 ml. per 30 ml. serum) to remove natural hemolytic antibody. After absorption the serum pool was distributed in 2 or 3 ml. portions in clean Pyrex glass ampoules which were promptly sealed and frozen in solid CO_2 . Shortly before use in an experiment an ampoule was opened and the contents diluted accurately with ice cold veronal-bicarbonate buffer.

Experimental Procedure.—Initially, attempts were made to use Ponder's method (10) of observing the rate of hemolysis directly in a photometer. The reaction mixture was placed in a photometric cuvette held at constant temperature by means of a water jacket through which distilled water kept at 37°C . was circulated. The red cells were maintained in uniform suspension by continuous stirring, and the lytic process was followed by measuring the light scattered at a 90° angle. It proved difficult, however, to secure precise, reproducible measurements with this arrangement because the photometric readings varied with the speed of motion of the red cells, due to the fact that the intensity of light scattering depends on the spatial orientation of the erythrocytes in respect to the light beam. As a remedy, stirring was discontinued and the cells allowed to come to rest before each reading, but in experiments requiring frequent readings this left too little time for the necessary stirring. Furthermore, the method of running the lysis inside a spectrophotometer is limited to one experiment at a time. As a result of these difficulties, the alternative procedure, described below, was adopted.

Ten ml. erythrocyte suspension, standardized spectrophotometrically to contain 5×10^3 cells per ml., and 10.0 ml. of diluted C' are mixed in a thoroughly cleaned⁶ 125 ml. Erlenmeyer flask suspended from a mechanical rocker⁷ in a water bath at $37^{\circ}\text{C} \pm 0.05^{\circ}$. After about 8 to 10 minutes' shaking to attain temperature equilibrium, 5.0 ml. of antibody dilution, also prewarmed to 37°C ., is added, and timing of the lytic reaction is begun. The mixture is shaken continuously during the entire experiment, except for interruptions of about 10 seconds during sampling. At stated time intervals, samples of 1.5 ml. are withdrawn by means of an accurately calibrated pipette and immediately delivered into and mixed with 3.0 ml. of an ice cold solution containing 0.12 M NaCl and 0.015 M sodium citrate, the latter salt serving to arrest lysis by binding⁸ of Mg^{++} and Ca^{++} (13). The 1.5 ml. pipettes used for sampling have delivery periods of about 10 seconds from mark to tip, and the time recorded for each sample is taken at the moment when one-half of the contents of the pipette has run out. The pipette is allowed to drain for a few seconds after the fluid has reached the tip, and is then blown out, in accordance with the procedure used for calibration. Efforts have been made to adhere to a

⁶ Pipettes, flasks, and test tubes were cleaned with dichromate-sulfuric acid mixture.

⁷ The Erlenmeyer flask is held in a short clamp attached to a horizontal rod which rotates back and forth through an angle of about 70 to 80° at a rate of 37 complete strokes per minute.

⁸ After addition of the sample withdrawn from the reaction mixture to the citrate solution, the concentrations of Mg^{++} , Ca^{++} , and citrate are 0.00017 M, 0.00005 M, and 0.01 M, respectively. Thus citrate is present in considerable excess.

uniform technique of sampling in order to keep the error of timing within ± 0.1 minute. After each sample, the pipette is rinsed once with 0.15 M NaCl solution, allowed to drain, and after blowing out the last drop the tip is wiped with dry filter paper.

The diluted samples are kept in ice water until the operator has time to place them in the centrifuge. They are spun for 10 minutes in the cold, the supernatant fluids are poured off, and the hemoglobin content of each supernate is determined spectrophotometrically at 541 $m\mu$.

Calculations.—Since the standardized cell suspension contains 5×10^9 erythrocytes per ml. the total dose of 10.0 ml. equals 5 billion cells. Upon addition of C' and antiserum the cells are diluted 2.5-fold. On admixture of citrate-saline with the samples withdrawn from the reaction mixture a further three-fold dilution occurs. Thus, the final dilution factor equals 7.5, and on this basis a completely lysed sample corresponding to lysis of the 5 billion cells in the entire reaction mixture yields an optical density reading of 0.68. Therefore, the number of cells decomposed in a partially lysed sample is calculated by multiplying the optical density by the factor $\frac{5 \times 10^9}{0.68} = 7.35 \times 10^9$.

Appropriate blanks are included in each experiment and data are corrected accordingly. As shown in the protocol for Table I, in experiments with excess C' the essential blank contains cells and C', but no antibody. On the other hand, experiments with excess antibody require a blank containing cells, antibody, and SA-buffer, or cells, antibody, and C' inactivated by heating at 56°C. for 50 minutes. Since it could be shown that C' heated under these conditions is entirely devoid of hemolytic activity, blanks containing cells, antibody, and SA-buffer are satisfactory and have therefore been used throughout.

It has been customary in studies on hemolysis to express results in terms of per cent lysis. This was not done in the present investigation because rather complex calculations would be necessary to correct the percentage values for the progressively increasing degree of lysis observed in the blank, and also because it seemed preferable to measure velocity of lysis in absolute terms. In expressing results in absolute numbers, as in the present experiments, it must be borne in mind, however, that complete hemolysis corresponds to 5 billion cells less the number of cells lysed in the blank. Depending on the magnitude of the blank, this value varied between 4.6 and 4.9 billion cells.

Protocols.—The complete record of a typical experiment is shown in Table I in order to furnish the details of measurement and calculation. As outlined in the following protocol the experiment consisted of three runs with antiserum pool B at dilutions of 1/20,000, 1/30,000, and 1/50,000,⁹ respectively, as well as three controls containing, respectively, cells alone, cells mixed with C', and cells mixed with antiserum diluted 1/20,000.

Flask.....	A	B	C	D	E	F
Standardized cell suspension, ml.....	10.0	10.0	10.0	10.0	10.0	10.0
C', diluted 1/30, ml....	0	10.0	0	10.0	10.0	10.0
0.2 per cent SA-buffer, ml.....	10.0	0	10.0	0	0	0

All flasks put simultaneously into water bath and prewarmed for 10 min.
Then added antiserum dilution or 0.1 per cent SA-buffer and started timing.

(continued on following page)

⁹ Throughout the paper these values represent the dilution of the 5.0 ml. portion of antiserum added to 10.0 ml. of cells and 10.0 ml. of C'. The final dilution would therefore be 5 times as high.

(Continued from preceding page)

Flask	A	B	C	D	E	F
0.1 per cent SA-buffer, ml.	5.0	5.0	0	0	0	0
Antiserum B, amount and dilution.	0	0	5.0 ml., 1/20,000	5.0 ml., 1/50,000	5.0 ml., 1/30,000	5.0 ml., 1/20,000
Time of addition of 0.1 per cent SA-buffer or antiserum.	0 min.	1.3 min.	8.2 min.	5.5 min.	6.9 min.	9.4 min.

The red cells were suspended in veronal buffer⁴ containing 0.1 per cent SA. Antiserum dilutions were also made in 0.1 per cent SA-buffer. C', lot 7, was diluted 1/30 in plain veronal buffer.⁴ Ten ml. of cell suspension and 10.0 ml. of C' dilution were mixed in each Erlenmeyer flask, except flasks A and C which received 0.2 per cent SA-buffer in place of C'. The flasks were simultaneously placed in the water bath at 37.0°C. and rocked for 10 minutes to attain constant temperature. Five ml. of prewarmed antiserum dilution (or 0.1 per cent SA-buffer for flasks A and B) was then added to each flask in the order, A, B, D, E, C, and F, and the time of addition noted. The sequence of addition was chosen so as to add the lowest dilution of antiserum last in order to compensate for the shorter lag period in reaction mixtures containing higher concentrations of antibody. Timing by means of a stop-watch was started upon addition of 0.1 per cent SA-buffer to flask A. The time readings for the final additions to the other flasks and for the withdrawal of samples were recorded as *absolute time* to be corrected to *net time* by subtraction of the absolute time at which the final addition to each flask was made (cf. bottom line in protocol).

The initial reading for the cell blank in flask A gave an optical density of 0.011, corresponding to initial spontaneous lysis of 1.6 per cent of the cells. During the 434 minutes which this experiment ran, this value rose to 0.017, or 2.5 per cent lysis, a negligible increase. The "cell + antiserum" blank in flask C gave an initial reading of 0.018 (2.6 per cent) and reached 0.023 (3.4 per cent) at the end of the run. While this mixture showed somewhat more initial lysis than the cell blank (A), the increase which occurred during the run was the same. The "cell + C'" blank in flask B gave an initial optical density reading of 0.023 and the difference between this value and the reading of 0.011 in flask A is accounted for by the color of the guinea pig serum used as C'. Reaction mixture B, however, showed a much larger increase during the run than either A or C. This is presumably due to traces of hemolytic antibody left in the C', even after repeated absorptions with packed sheep erythrocytes. Unabsorbed C' yields much higher blanks and is therefore unsuitable.

The optical density values for flask B were plotted against the absolute time readings and a smooth curve was drawn through the points by inspection. For correction, the blank values at the appropriate times were read from the curve and subtracted from the optical density readings of the samples from flasks D, E, and F. These corrections were made on the basis of *absolute*, rather than *net* time because the lytic action due to guinea pig serum alone starts as soon as the flasks are placed in the water bath and is therefore not affected by the staggered addition of the antiserum dilutions. The blanks represented by flasks A and C were not used in calculating the results, but were included solely for the purpose of checking the stability of the cells. The corrected optical density values were converted into the number of cells lysed by multiplying with the factor 7.35×10^7 , as explained above.

Data from Table I are represented in Fig. 1 by curves 1, 2, and 3. Results of another experiment with antiserum B, diluted 1/15,000 and 1/10,000, are shown in curves 4 and 5.

The experiment shown in Fig. 2 was designed to demonstrate the effective-

TABLE I
Velocity of Hemolysis with Excess C' and Limited Amounts of Antibody

Flask...	A			B			C			D			E			F		
Sample No.	T	T'	O.D.	T	T'	O.D.	T	T'	O.D.	T	T'	O.D.	T	T'	O.D.	T	T'	O.D.
1	11.7	11.7	0.011	13.3	12.0	0.023	15.2	7.0	0.018	16.8	11.3	0.035	18.4	11.5	0.033	20.0	10.6	0.087
2	32.3	32.3	0.011	34.1	32.8	0.025	35.8	27.6	0.020	37.4	31.9	0.073	39.2	32.3	0.158	40.8	31.4	0.290
3	50.5	50.5	0.012	53.0	51.7	0.027	54.9	46.7	0.021	57.5	52.0	0.103	59.3	52.4	0.223	61.4	52.0	0.377
4	73.0	73.0	0.012	74.2	72.9	0.028	75.7	67.5	0.021	77.5	72.0	0.128	79.3	72.4	0.262	81.3	71.9	0.423
5	108.7	108.7	0.012	110.8	109.5	0.031	112.6	104.4	0.022	114.6	109.1	0.162	116.6	109.7	0.315	118.7	109.3	0.473
6	141.8	141.8	0.013	143.4	142.1	0.032	145.2	137.0	0.022	147.1	141.6	0.183	149.2	142.3	0.345	150.9	141.5	0.500
7	178.8	178.8	0.014	180.3	179.0	0.034	182.0	173.8	0.022	183.7	177.2	0.202	185.9	179.0	0.367	188.0	178.6	0.517
8	210.7	210.7	0.014	212.2	210.9	0.035	213.8	205.6	0.022	215.7	210.2	0.216	217.4	210.5	0.383	219.1	209.7	0.528
9	254.7	254.7	0.014	256.3	255.0	0.036	257.9	249.7	0.023	259.7	254.2	0.235	261.4	254.5	0.406	263.1	253.7	0.545
10	304.0	304.0	0.014	305.7	304.4	0.038	307.5	299.3	0.023	309.1	303.6	0.248	310.8	303.9	0.422	312.4	303.0	0.555
11	357.8	357.8	0.017	359.4	358.1	0.038	361.3	353.1	0.023	363.5	358.0	0.263	365.1	358.5	0.432	367.4	358.0	0.558
12	434.2	434.2	0.017	435.7	434.1	0.042	437.6	429.4	0.023	439.3	433.8	0.281	441.1	434.2	0.450	442.9	433.5	0.577

T, absolute time.

T', net time.

O.D., optical density.

O.D.', net optical density.

No., number of cells lysed in billions.

ness of sodium citrate in arresting the hemolytic process. Duplicate determinations were set up with antiserum A at a dilution of $1/10,000^9$ and C'

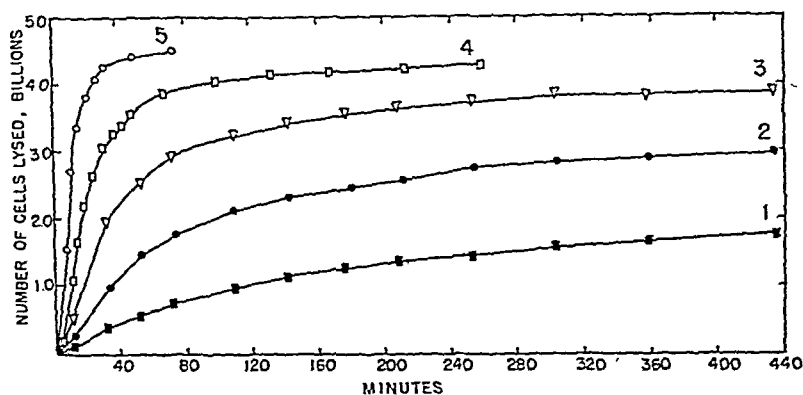


FIG. 1. Course of hemolysis with constant excess of C' ($1/30$), and varying dilutions of antiserum pool B.

Curve No.....	1	2	3	4	5
Antiserum dilution.....	$1/50,000$	$1/30,000$	$1/20,000$	$1/15,000$	$1/10,000$

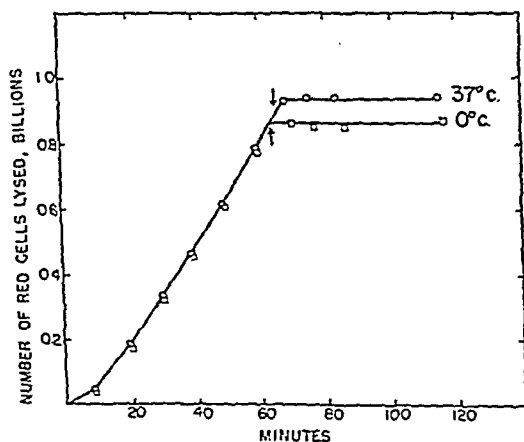


FIG. 2. Effectiveness of sodium citrate in stopping hemolysis. Arrow indicates time of addition of citrate-saline.

diluted $1/30$. A blank control containing cells and C' but no antibody was also run in duplicate. After withdrawal of 6 samples of 1.5 ml. from each of the flasks (*i.e.*, 9.0 ml. total withdrawn) another 6.0 ml. was removed and discarded, in order to reduce the volume of each reaction mixture to 10.0 ml. Immediately afterwards 20.0 ml. portions of ice cold citrate-saline were added

to one of the determinations and one of the blanks, the mixtures were transferred from the 37° water bath to an ice water bath, and kept there with manual agitation every few minutes. To the other determination and blank 20.0 ml. portions of citrate-saline prewarmed to 37°C. were added and the mixtures kept in the 37°C. water bath with continuous rocking. Sampling was then resumed and the portions removed were immediately centrifuged in the cold to separate the intact cells.

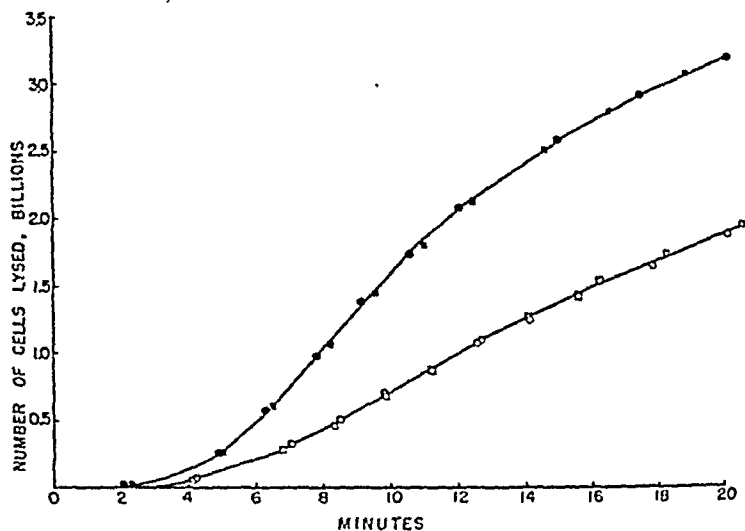


FIG. 3. Effect of varying the intensity of agitation during hemolysis. C' present in constant excess. Hemolytic antiserum (Lederle commercial amboceptor) diluted 1/10,000 (solid symbols), and 1/15,000 (open symbols). Circles and squares represent rocking through arcs of 70° and 20°, respectively.

The results indicate that addition of citrate-saline arrests hemolysis completely, and that in conjunction with chilling its action is practically instantaneous.

It appeared advisable to determine whether the intensity of agitation affects the rate of hemolysis (Fig. 3). Duplicate runs were made with C', lot VI, diluted 1/30 (*i.e.* an excess) and a hemolytic antiserum from Lederle Laboratories, used in 2 dilutions, *viz.* 1/10,000 and 1/15,000. One set of determinations including the blank was shaken in the usual manner with an angular excursion of 70°, while the other set was shaken with an excursion of 20°.

Since results were identical under both conditions the intensity of agitation does not represent a critical factor, provided, of course, that it is adequate to maintain the cells in uniform suspension.

In another experiment the rate of shaking was increased to 46 strokes per

minute with an angular excursion of about 70 to 80°, but the velocity of hemolysis was not affected.

The experiment presented in Fig. 4 was carried out with excess C' (dilution 1/30), and limited amounts of antiserum pool A, with dilutions ranging from 1/2500 to 1/30,000. While of the same type as the experiment with antiserum pool B, shown in Fig. 1, the characteristic shapes of the velocity curves are dissimilar, indicating that different antisera such as pools A and B may exhibit diverse kinetic behavior. In some of the experiments incorporated in this series, C', lot V, was used, while others were performed with lot VI, but no sig-

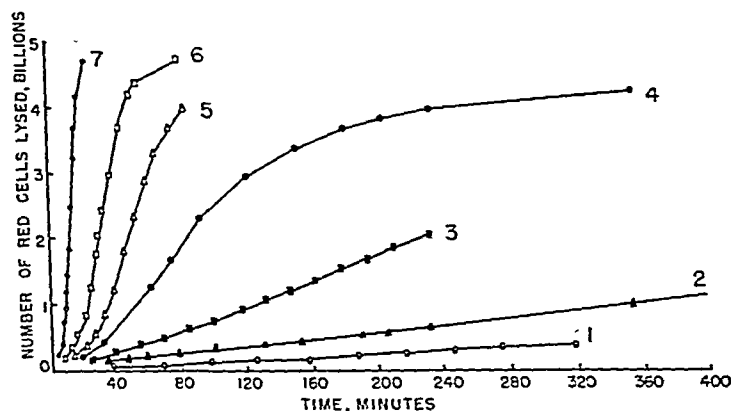


FIG. 4. Course of hemolysis with constant excess of C' and varying dilutions of antiserum pool A.

Curve No.	1	2	3	4	5	6	7
Dilution	1/30,000	1/20,000	1/14,000	1/10,000	1/7000	1/5000	1/2500

nificant difference was noted when these 2 lots of C' were compared at the same dilutions of antibody.

While it has not yet been possible to subject the velocity curves in Figs. 1 and 4 to mathematical analysis, a comparison of the curves obtained with different dilutions of antiserum A (Fig. 4) has been made on the basis of the maximal slopes. These were measured from the graph in the vicinity of the inflection point and plotted against the reciprocal of the antiserum dilution to yield the plot of velocity vs. relative concentration of antibody shown in Fig. 5. As may be seen the velocity is a linear function of concentration over most of the range except at very low concentrations of antibody. It is also evident that the speed of lysis could serve as a sensitive and precise measure of antibody concentration.

The experiment shown in Fig. 6 was carried out with an excess of antibody

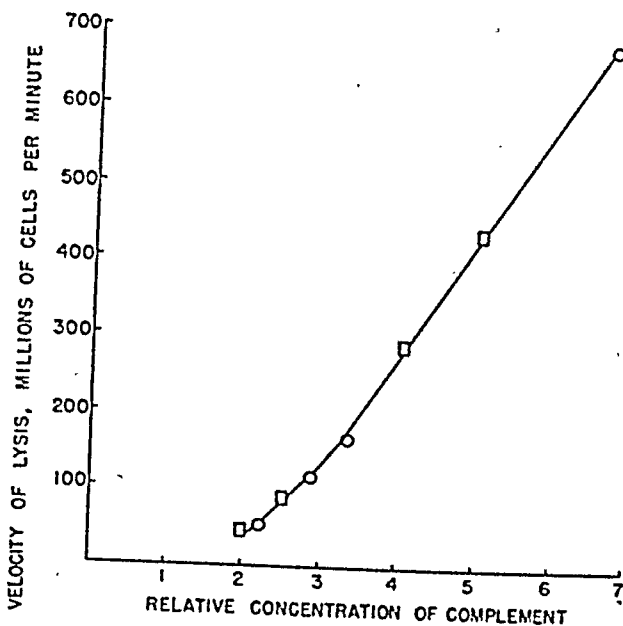


FIG. 8. Velocity of lysis as a function of the concentration of C' . Data shown by circles were taken from Fig. 7, while the squares represent another experiment.

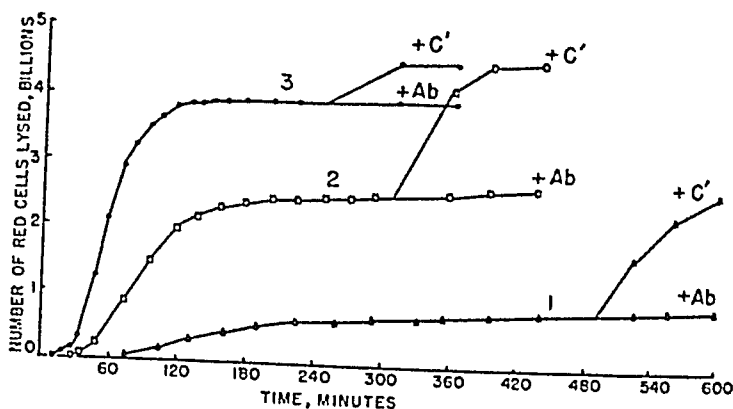


FIG. 9. Course of hemolysis with a constant, but limited amount of antibody (antiserum A diluted 1/5000) and varying dilutions of C' . The experiment was set up with 1.5 times the usual amounts of reagents yielding a total of 37.5 ml. of reaction mixture. The two branches of each curve indicate the course of hemolysis after splitting the reaction mixture and addition of the following amounts of antibody or C' to the respective portions.

Curve No.....	1	2	3
Dilution of C' used initially.....	1/400	1/200	1/100

Sampled until hemolysis ceased; then split each reaction mixture into 2 equal portions

Volume of each of the portions, ml.....	5.5	6.0	4.5
Undiluted C' added after split, ml.....	0.011	0.024	0.018
1/200 dilution of antiserum added after split, ml.....	0.044	0.048	0.036

experiments hazardous. As far as observations go, it is therefore evident that hemolysis proceeds without stop even with minute amounts of antibody, provided C' is present in excess.

To confirm this finding, an experiment, shown in Fig. 9, was set up with relatively limited amounts of both antibody and C'. When hemolysis had stopped the contents of each flask were divided in half. One portion was mixed with additional antiserum while the other received additional C'. As a result, lysis was resumed upon addition of more C', but not upon supplying more antibody. This indicates that lysis had stopped due to exhaustion of the available C'.

Additional Experimental Data.—Antiserum pools A and B were analyzed for total antibody content by the procedure of Heidelberger and Treflers (17). Hemolytic antibody titers were calculated from the kinetic data in Figs. 1 and 4, on the basis of an incubation period of 80 minutes. Agglutination titers were determined in the usual manner. The results of these measurements are given

TABLE II
Summary of Properties of Antiserum Pools A and B

	Total antibody nitrogen	Hemolytic titer	Agglutination titer
	mg.		
Antiserum pool A.....	0.61	5000	1000
Antiserum pool B.....	0.20	10,000	400

in Table II. On the basis of 0.20 mg. total antibody N per ml. in antiserum B, and a molecular weight of 900,000 for hemolytic antibody (18), it can be calculated from curve 1 in Fig. 1 that 84 billion molecules of antibody effected lysis of 1.75 billion cells in 440 minutes. This is equivalent to 48 molecules of antibody per red cell, a value of the same magnitude as that calculated by Brunius (24), but much lower than that of about 500 estimated by Heidelberger, Weil, and Treflers (19).

RESULTS AND DISCUSSION

Measurements of the rate of hemolysis can be made with a precision of ± 5 per cent, comparable to that attained in static analyses (12, 13), provided experimental conditions are adjusted so that the speed of lysis is within the range of about 10 to 500 million cells per minute. In experiments with an excess of C' the lower limit is imposed by the lytic speed of about 0.5 million cells per minute usually observed in the blank containing cells and a 1/30 dilution of C'. On the other hand, when only small amounts of C' are used the blank is practically negligible, but in this case a lower limit for the accurate determination of the lytic rate is set by the early cessation of lysis upon exhaustion of the limited

amount of C' . The upper limit is due to the fact that samples cannot be taken more frequently than about once every minute, and that timing of the delivery of samples into the citrate-saline solution is subject to an uncertainty of about 0.1 minute. Since earlier investigations, in which centrifugation served as a means of arresting hemolysis, suffered from a timing error of at least 1 or 2 minutes, the use of citrate-saline constitutes a substantial improvement.

It is evident from the duplicate experiments shown in Figs. 2 and 3 that a high degree of reproducibility can be achieved in experiments carried out with the same suspension of red cells. With different lots of red cells agreement is not always satisfactory due to variations in lytic susceptibility among cells from different sheep and of different age. If comparable results are desired in runs carried out at different times, it is therefore advisable to use erythrocytes derived from the same animal, and not differing too widely in respect to the period of storage in the Alsever solution. Studies on these sources of error are still in progress.

In agreement with earlier investigations (10, 11) the present experiments show that plots of the degree of hemolysis against time yield more or less S-shaped curves, which vary in their characteristics depending on the respective concentrations of the lytic agents. According to Ponder (10), the S-shaped nature of the velocity curves is assumed to be due to the heterogeneity of red cells in respect to their susceptibility to lysis (20). Thus, an S-shaped integral distribution function would form the fundamental pattern of all the velocity curves, but modifications due to various kinetic factors involving the concentrations of erythrocytes, antibody, and C' , as well as their respective speeds of interaction, are superimposed upon the basic pattern to yield the variety of curves shown in Figs. 1, 4, 6, and 7. While the mechanism of the hemolytic process is not as yet understood sufficiently well to permit formulation of a quantitative kinetic theory, a limited qualitative evaluation of the present findings can be attempted.

The most striking result of the present study is the recognition that the kinetics of hemolytic systems containing excess antibody and limited C' (Figs. 6 and 7) differ profoundly from those of systems in which C' is present in excess and antibody is limited (Figs. 1 and 4). Thus, with C' as the limiting factor, the hemolytic reaction runs for a period of only about 40 to 80 minutes, and then stops completely. Since it has been shown that C' is used up or destroyed during hemolysis (9), the lytic process presumably comes to a standstill when the available supply of C' has been exhausted. The number of cells lysed at the end point varies with the amount of C' according to a function such as the well known equation of von Krogh (21).

On the other hand, when C' is present in excess and antibody is the limiting factor, as in Figs. 1 and 4, the velocity curves have a catalytic appearance, resembling those obtained in enzymic reactions. Thus, in experiments with anti-

serum B (Fig. 1), readings were taken for about 440 minutes and progressively increasing hemolysis was noted. Although the velocity became very low toward the end of that period, lysis did not cease completely. The decline in velocity was not due to lack of C' since it could be shown that the system still contained excess C' at the end of the 440 minutes. With antiserum A (Fig. 4) the catalytic appearance of the curves is especially pronounced since velocities did not diminish appreciably until a substantial part of the red cell substrate had been destroyed.

Since an end point is not reached, at least within practicable periods of observation, any relation between the number of cells lysed and the amount of antibody employed must involve time as a variable. Titrations of hemolytic antibody should therefore be based on determinations of the number of cells lysed in unit time; *i.e.*, measurements of velocity. Thus, the problem of assaying the activity of hemolytic antibody is essentially similar to that of determining the activity of an enzyme.

In view of the initial lag period exhibited by the hemolytic velocity curves, analysis for antibody, however, cannot be made in terms of initial reaction velocity, as in the determination of enzymes. Ideally, the kinetic velocity constant proper should be used as a measure of antibody activity, but, lacking a kinetic equation, this is not possible at present. Instead, the optimal velocity of each curve, *i.e.* the slope at the inflection point, can be employed as an empirical index of the activity of hemolytic antibody. As shown in Fig. 5 the relation between maximal velocity and antibody concentration is linear, except at very low concentrations of antibody. A similar plot, relating velocity and concentration of C' , is shown in Fig. 8.

The experiments plotted in Figs. 1, 4, 6, and 7 represent extreme conditions in respect to excess of either C' or antibody. In view of the startling results, it seemed of interest to study the course of hemolysis in systems of an intermediate type; *i.e.*, not containing an excess of either C' or antibody. Such an experiment is shown in Fig. 9, and it can be seen that the reaction stopped at different levels of hemolysis depending on the amount of C' used. In order to ascertain why the lytic process had stopped, each reaction mixture was split in half upon cessation of lysis, and additional antibody was added to one portion, while the other received more C' . The results show that increasing the supply of antibody had no effect, while lysis was promptly resumed when fresh C' was introduced, indicating that the cessation of lysis was due to exhaustion of C' . This is borne out especially well with curve 1, the experiment with C' diluted 1/400. In this run, the amount of C' added after cessation of lysis was chosen so as to raise the concentration of C' to that initially present in the experiment represented by curve 2. As a result, the speed of reaction on resumption of lysis in Experiment 1 became about the same as that observed initially in Experiment 2. Since the same amount of antibody was used in all three

runs represented in Fig. 9, it can be concluded that the effective concentration of antibody was unaltered even after 4 hours of continuous action.

It is therefore evident that hemolytic antibody, in sharp contrast to C' , possesses the ability to act for long periods of time without suffering exhaustion. However, different antisera do not possess this capacity to the same degree. Thus, comparison of Figs. 1 and 4 shows that antiserum A is slower in getting lysis under way, but maintains a high rate of lytic activity longer than antiserum B. As a result, a given dilution of antiserum B, such as, for example, curve 3 in Fig. 1, may initially produce more hemolysis than a comparable dilution of antiserum A (e.g., curve 5 in Fig. 4), but after a while the action of B becomes slower than that of A, the curves cross, and antiserum A appears to be more potent. Three other hemolytic antisera, which have been studied by the kinetic method, have shown similar variations, one being analogous in behavior to A, another resembling B, and the third displaying an intermediate type of kinetics. In addition to these differences, it has been found (*cf.* Table II) that while the absolute antibody contents of antisera A and B are in fair agreement with their agglutination titers, they bear no relation to the hemolytic titers. In the studies of Heidelberger and Treffers (17) discrepancies between hemolytic activity and antibody content were also noted, and it was pointed out that antisera to sheep erythrocytes contain a multiplicity of antibodies reactive with various antigenic constituents of the red cell. Perhaps only one, or a few of these antibodies possess hemolytic activity. Thus, the differences between antisera A and B shown in Table II might be due to a higher proportion of non-hemolytic antibody in antiserum A. On the other hand, the dissimilar kinetic behavior of antisera A and B cannot be explained on this basis.

In order to interpret the catalytic character of the velocity curves in Figs. 1 and 4, as well as the differences between them, it is necessary to consider how antibody and C' function in the hemolytic process. According to the classical concepts, C' is assumed to be the lytic agent proper, while antibody merely serves to fix C' at its site of action; *i.e.*, upon the surface of the red cell (9, 10). Since C' is consumed or destroyed during hemolysis, the catalytic character of the velocity curves in systems containing C' excess, indicates that progressively increasing amounts of C' are fixed and utilized as lysis proceeds. This view derives support from recent quantitative studies on the fixation of C' (16), in which it was shown that uptake of C' by antigen and antibody at 37°C. increases continually with time. Plots of the velocity of C' fixation in reference 16 closely resemble those for the hemolytic reaction in the presence of excess C' . On the other hand, the concept of continuous utilization of C' during hemolysis is in conflict with Ponder's statement (10) that the fixation of C' by sensitized cells is completed within less than one-third of the latent period; *i.e.*, the time elapsed prior to onset of hemolysis.

If the continuous utilization of C' involves uptake of progressively increasing

amounts of C' substance by the sensitized erythrocytes, steric limitations should eventually terminate the hemolytic process, unless there exists a mechanism for the release of expended C'. It is therefore pertinent to consider whether C' fixation is a reversible process. While there is no direct evidence, indirect support can be derived from the fact that the union between antigen and antibody has been shown to be reversible under appropriate conditions (22, 23). Since fixation of C' results from the combination of antigen with antibody, dissociation of the immune complex should be accompanied by release of C'.

It therefore appears possible that a molecule of hemolytic antibody could combine with a red cell, perform its lytic function in cooperation with C', and eventually dissociate to become available for action at some other site of the same or of a different red cell. Thus, the molecules of antibody would go through a cycle enabling them to react over and over again, and produce progressively increasing hemolysis, provided a sufficient supply of C' is available. Experiments on this hypothesis are in progress.

It is interesting to speculate on the significance of the present findings in respect to the hypothesis that immune hemolysis is an enzymatic process. The recent demonstration that Mg^{++} is an essential cofactor in the hemolytic reaction (13) has lent new support to this concept since Mg^{++} is also necessary to many enzyme systems. Such speculations have usually revolved about C' as the enzyme, but the present data show that in its kinetic behavior antibody resembles an enzyme more closely than does C'. The exceedingly small number of antibody molecules required for the lysis of a single red cell (48 molecules in the case of antiserum B acting for 440 minutes) would also support this view. Furthermore, just as enzyme and substrate unite as the result of specific structural affinity, antibody and red cells combine for the same reason. If this be true, the function of C' might be that of a cofactor, possibly an energy donor, which would permit the hemolytic antibody to exercise enzyme-like activity.

SUMMARY

A quantitative method for the study of the kinetics of immune hemolysis is described.

In the presence of excess C' and limited amounts of antibody the kinetics of hemolysis resemble those of enzymatic processes.

Assays of hemolytic antibody should therefore be based on velocity measurements rather than end point titrations.

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THE PRODUCTION OF A PERSISTENT ALTERATION IN INFLUENZA VIRUS BY LANTHANUM OR ULTRAVIOLET IRRADIATION

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The capacity of influenza virus to combine with and agglutinate red blood cells and thereafter to be eluted from them (1, 2) has stimulated numerous investigations. As a result of early work it was assumed that adsorption of the virus to RBC and subsequent dissociation of the complex were closely associated phenomena and it was suggested that the reactions could be considered as analogous to those between an enzyme and its substrate (2). Shortly after the discovery that influenza virus could cause hemagglutination it was found (2) that two different virus strains, *i.e.* Lee and PR8, showed different rates of elution from RBC. Recently it was shown (3) that by heating the virus the elution phenomenon could be almost completely abolished without affecting the capacity of the agent to combine with RBC and cause hemagglutination. Heat, however, inactivates the virus; the property of multiplication in susceptible hosts is lost and probably other properties also are altered. It was decided to investigate the elution of influenza virus from RBC more thoroughly and particularly to attempt to find means whereby the rate of elution of the virus could be altered without destroying the capacity of the agent to induce infection.

The results obtained in the present study provide evidence that it is possible by means of certain physical or chemical agents, *i.e.* treatment with lanthanum acetate or ultraviolet irradiation, to produce a marked alteration in the elution rate of the Lee strain of influenza virus without causing demonstrable alterations in the other properties of the virus tested. It will be shown that strains modified by the procedures employed retained their altered state on serial passage in the chick embryo in the absence of the agent which originally effected the alteration.

Materials and Methods

Viruses.—The PR8 strain (4) of influenza A virus and the Lee strain (5) of influenza B virus were used in this study. Both strains had previously been passed many times in mice and in chick embryos. Between experiments allantoic fluids infected with either virus were stored frozen in sealed ampoules in a CO₂ storage cabinet at -70°C .

Chick Embryos.—White Leghorn eggs were incubated at 39°C . for 9 to 12 days. Embryos of the desired age were inoculated into the allantoic sac through a paraffin-sealed hole drilled in the shell. The inoculum consisted of 0.1 cc. of infected allantoic fluid diluted 10^{-2} in sterile

broth unless otherwise stated. After inoculation chick embryos were incubated for 48 hours at 35°C. and candled daily. Embryos which died were discarded. Eggs containing living embryos then were held at 4°C. for approximately 2 hours and thereafter the allantoic fluid was harvested in the usual manner. Fluids which gave positive hemagglutination with chicken RBC were pooled and used as virus source material. Depending upon the experiment the pooled allantoic fluids were stored either at 4°C. or at -70°C.

Virus Titrations.—Hemagglutination titrations were done according to the technique described by Hirst (6). Serial twofold dilutions of allantoic fluid pools were prepared in saline buffered at pH 7.2. To 0.4 cc. of each dilution was added 0.4 cc. of a 1 per cent suspension of washed chicken RBC. The tubes were shaken and the degree of hemagglutination recorded after 1 hour at room temperature. The patterns of the sedimented erythrocytes were graded from 4+ to \pm in the customary manner and the end point was taken as the highest dilution which gave a 2+ pattern.

Virus infectivity titrations in chick embryos were done by the intra-allantoic technique (7) in 9 to 12 day embryos. Serial tenfold dilutions of allantoic fluid pools were prepared in sterile broth. A group of 4 embryos was inoculated with each dilution; each embryo received 0.1 cc. Allantoic fluid was harvested from each embryo after 48 hours' incubation at 35°C. and tested by the hemagglutination technique. The end point was taken as the highest dilution which induced demonstrable infection in 2 or more embryos in a group.

Virus infectivity titrations in mice were carried out by the intranasal technique. Serial tenfold dilutions in sterile broth were employed. A group of 6 Swiss mice was used for each dilution. The procedure was identical to that previously described (8) and the 50 per cent maximum score end point (M.S.50) was used.

Immune Serum.—Rabbits were immunized by the intravenous injection of infected allantoic fluid pools. A single injection of 10 cc. of undiluted fluid was given. Serum was obtained 14 to 21 days later and was stored without preservative at 4°C. Immediately before use in hemagglutination-inhibition tests serum was diluted 1:2 with saline and heated at 65°C. for 30 minutes. This procedure markedly reduces the so called non-specific inhibitory capacity of serum but does not significantly diminish the antibody titer of immune serum (9, 10).

Identification of Virus Strains.—Serological identification of the virus strains studied was carried out by means of the hemagglutination-inhibition technique (6). Serial twofold dilutions of infected allantoic fluid were prepared in saline. Each dilution was mixed with constant amount serum which was diluted so as to contain a quantity of antibodies just capable of inhibiting hemagglutination by 256 units of virus. To each mixture was added an equal quantity of a 1 per cent suspension of chicken RBC. The end point was taken as the highest dilution of infected allantoic fluid which caused 2+ hemagglutination. As a routine in hemagglutination-inhibition tests both anti-PR8 and anti-Lee immune as well as normal rabbit serum were employed.

Determination of Eluted Virus.—Inasmuch as the chief objective of this study was to investigate possible alterations in the capacity of influenza virus to dissociate from RBC after combination with them, it was essential to devise a technique by means of which both the rate of elution of the virus and the quantity of virus eluted could be determined. The following technique was found to be suitable: The RBC in 5 cc. of a 1 per cent suspension were packed by a few minutes' centrifugation at 4,000 r.p.m. The supernate was poured off and discarded. To the packed cells 1 cc. of an infected allantoic fluid pool was added and the mixture was shaken vigorously. Small volumes were employed to facilitate very rapid sedimentation of the RBC. Only pools with hemagglutination titers of 1:1024 or more were used. The final concentration of erythrocytes in the mixture was 5 per cent. The mixture was held at room temperature for 10 minutes in order for virus-erythrocyte combination to occur. In every instance hemagglutination occurred almost immediately after the mixture was made and the agglutinated cells settled very rapidly. At the end of 10 minutes the mixture was reshaken

and the cells sedimented by centrifugation at 4,000 R.P.M. for approximately 1 minute. The supernate was poured off and its hemagglutination titer determined subsequently. Immediately 1 cc. of buffered saline was added to the packed cells; this mixture was shaken vigorously and then held at room temperature. Identical cycles of centrifugation, removal of supernate, and resuspension of the sedimented cells in 1 cc. of fresh saline were carried out 30, 60, 90, 120, and 180 minutes later. Each supernate was kept so that its hemagglutination titer could be determined. In certain experiments even shorter periods were employed in each successive elution step.

This technique has made it possible to obtain fairly reproducible virus elution curves relative to time and has facilitated the present study. It should be pointed out that the resuspension of the agglutinated cells in fresh saline after each centrifugation makes it possible to determine the dissociation of relatively small quantities of virus during each time interval which is technically very difficult if the initial cell-virus mixture is retained and supernate aliquots are removed successively. It should be mentioned also that it is important to employ a relatively large amount of virus so that all RBC "receptors" rapidly combine with the agent; *i.e.*, are saturated. Otherwise, virus particles which dissociate from one RBC could promptly combine with another and only appear in the supernate subsequently thus leading to an inexact estimation of elution rate. It was found that under the conditions of these experiments allantoic fluid pools with hemagglutination titers of 1:1024 or more consistently saturated the quantity of cells employed as judged by the finding that demonstrable amounts of virus remained unadsorbed 10 minutes after the mixtures were prepared.

Ultraviolet Irradiation.—In all experiments the same ultraviolet source and distance were used. The lamp, which was kindly provided by Dr. George I. Lavin, was a "cold arc" resonance mercury lamp which operated at 15,000 volts A.C. The emission spectrum showed one main line at 2537 Å. Irradiation of infected allantoic fluid pools was performed in an open Petri dish with an inner diameter of 5 cm. Five cc. of fluid in this dish gave a depth of 5 mm. The distance from the fluid to the disc-shaped coil (diameter = 8 cm.) of the lamp, which was parallel to the fluid surface, was 9 cm. Irradiation was carried out for periods ranging from 15 to 60 minutes. Allantoic fluids were not dialyzed before irradiation.

Treatment with Lanthanum.—Lanthanum acetate (LaAc_3) was used for the treatment of infected allantoic fluid pools. The amorphous compound was dissolved in distilled water. Two stock concentrations, 0.1 and 0.01 M, were employed and the pH of the solutions used varied only between 6.5 and 7.0. When tested intra-allantoically in chick embryos, it was found that injections of 0.5 cc. of 0.1 M solutions did not kill the embryos. Solutions of LaAc_3 were mixed with equal quantities of infected allantoic fluid pools so as to give final concentrations ranging from 0.005 to 0.00005 M. The mixtures were held at 4°C. for 3 to 5 hours. Flocculent precipitates developed promptly. The precipitate was removed by centrifugation and discarded. The supernate was employed and excess lanthanum contained in it was precipitated by the addition of sufficient Na_2HPO_4 to give 0.5 M. This was done because lanthanum acetate even in very low concentrations causes agglutination of chicken RBC.

EXPERIMENTAL

Elution of Lee and PR8 Strains from RBC.—In order to determine whether persistent alterations in the rate of elution of influenza virus from RBC could be produced by experimental procedures, it was first necessary to devise a technique by means of which relatively small quantities of eluted virus could be measured. It was found that, when the RBC were sedimented from the mixture at fixed time intervals and resuspended in fresh saline after each centrif-

ugation, it was possible to measure with fair reproducibility the amount of virus which was eluted during each successive time interval. It appeared that in this manner the information desired could be obtained.

A number of experiments were carried out to determine the rates of elution of the Lee and PR8 strains from chicken RBC under these conditions. Hirst (2) showed previously, under different experimental conditions, that the Lee strain was eluted more rapidly than the PR8 strain.

The stepwise technique employed for the determination of the rate of elution of influenza viruses from RBC is described in detail above. In the present experiments successive 30 minute elution intervals were employed.

The results of a series of such experiments with a number of allantoic fluid pools containing either the Lee or the PR8 strain are presented in summary form in Table I. It can be seen that elution of the Lee strain is characterized by very rapid dissociation during the first 30 minute step and thereafter during successive 30 minute intervals by progressively less rapid dissociation. It is evident that after the second step (70 minutes) only very small quantities of virus were released into the supernatant fluids. The progressively decreasing rate of elution becomes especially evident when the percentage of virus eluted during each step is calculated relative to that combined with the RBC at the beginning of each step. As is shown in Table I, this quantity diminishes rapidly from an average value of 27.3 per cent during the first 30 minute step to no more than 0.3 per cent during the final 60 minute step.

The elution of the PR8 strain showed a very different pattern. Only relatively small amounts of virus were released from the RBC during each 30 minute interval and at no time did the titer of the supernates reach a high level as was the case with the Lee strain during the first 30 minute step. Moreover, it will be noted that the percentage of combined virus which was eluted during each step remained almost constant. This indicates that with the PR8 strain the rate of elution did not vary significantly during the time interval studied. In Figs. 1 and 2 the amounts of each strain which were eluted in successive steps are presented graphically. The logarithms of the geometric mean of the hemagglutination titers of the supernates are plotted against the time after the virus was mixed with RBC.

Similar experiments in which the supernatant fluid was changed at shorter intervals were carried out with both the Lee and PR8 strains. In every instance analogous results were obtained. Although it was possible by employing very short elution periods, *e.g.* 5 minutes or less, to obtain even more rapid elution rates with the Lee strain, it was not possible to increase the rate of elution of the PR8 strain as can be seen from the data shown in Fig. 3.

It is apparent that a number of factors might be responsible for the results obtained with the Lee strain. However, in the light of the findings presented

below, it seems of most interest to consider the possibility that the progressively decreasing rate of elution obtained with the Lee strain as not with PR8, may be an indication of an inhomogeneous population of Lee virus particles in infected allantoic fluid.

Effects of Lanthanum Acetate upon Influenza Viruses.—Hammarsten, Hammarsten, and Teorell (11) showed that lanthanum reacts with nucleic acids to

TABLE I
Results of Stepwise Virus Elution Experiments with Lee and PR8 Strains

Virus strain	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer* of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	9	min. 0 (Control)	8192	1024	2757	per cent —
"	"	10	256	16	34	—
"	"	40	2048	256	745	27.3
"	"	70	512	64	161	8.1
"	"	100	256	16	32	1.7
"	"	130	32	4	10	0.5
"	8	190	8	4	5	0.3
Total virus eluted.....					953	37.9
PR8	5	0 (Control)	4096	1024	2665	—
"	"	10	32	16	28	—
"	"	40	128	32	64	2.4
"	"	70	128	64	112	4.3
"	"	100	256	32	74	3.0
"	"	130	128	32	56	2.3
"	"	190	128	32	97	4.2
Total virus eluted.....					403	16.2

* Expressed as the reciprocal.

form an insoluble complex. It seemed possible that lanthanum could also react with influenza viruses and probable that, if it did, it might cause some alteration of the agents. This possibility was investigated.

When an aqueous solution of lanthanum acetate was added to normal allantoic fluid, a flocculent precipitate developed. The more concentrated the LaAc_3 solution, the more abundant was the precipitate. It was found that even in high dilution, e.g. 10^{-5} to 10^{-6} M, LaAc_3 caused agglutination of chicken RBC. Consequently, it was impossible to determine the hemagglutination titer of the virus in the presence of free lanthanum. When, however, a few

drops of 5 M Na_2HPO_4 solution were added to the mixture, the excess of LaAc_3 was precipitated and satisfactory virus hemagglutination titrations could be performed.

The addition of LaAc_3 to allantoic fluids containing influenza virus caused a prompt reduction in the hemagglutination titer of the virus and the reduction in titer appeared to be proportional to the concentration of lanthanum. Thus, a concentration of 0.0001 M did not

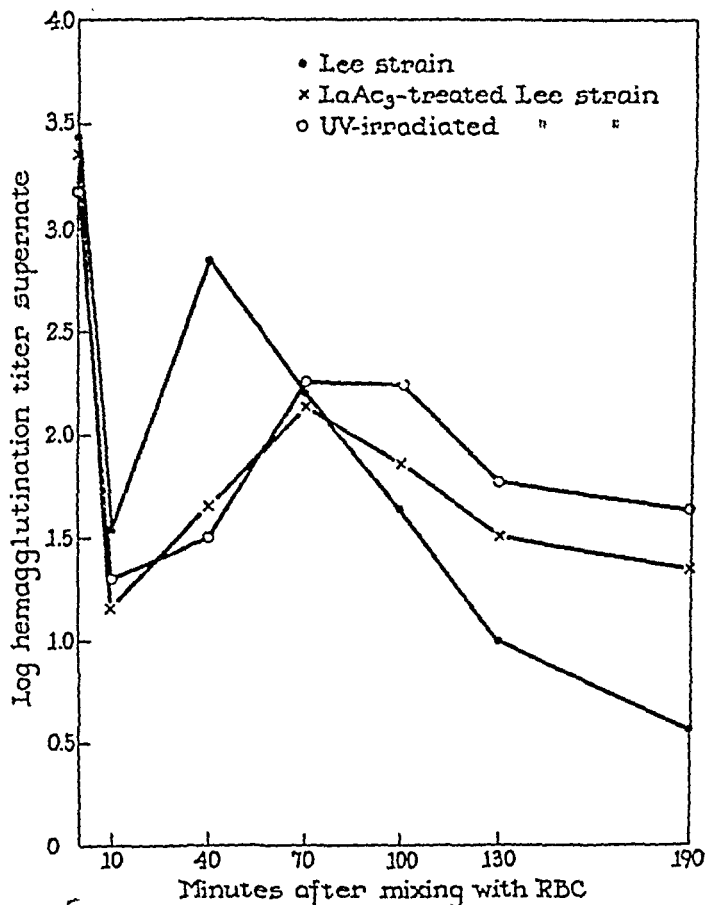


FIG. 1. Stepwise elution curves obtained with the Lee strain as well as with LaAc_3 -treated and ultraviolet-irradiated strains derived therefrom. The logarithm of the geometric mean of the hemagglutination titers of successive supernates (*cf.* Tables I, II, and IV) is plotted against the time at which the supernate was removed.

demonstrably affect the hemagglutination titer, concentrations of 0.01 to 0.001 M decreased the titer 100- to 1000-fold, while a concentration of 0.1 M abolished the hemagglutination reaction completely. The reduction in titer occurred immediately after the lanthanum was added and prolonged treatment, *e.g.* 10 hours, did not lead to any further decrease in titer.

The effect of LaAc_3 upon the infectivity of influenza virus also was studied. It was found that a mixture of equal parts of allantoic fluid infected with the

Lee strain and a 0.01 M solution of LaAc_3 was capable of infecting chick embryos. The virus infectivity titer of such a mixture, however, was lower by about 3 logarithmic units than that of the untreated allantoic fluid. Prolonged

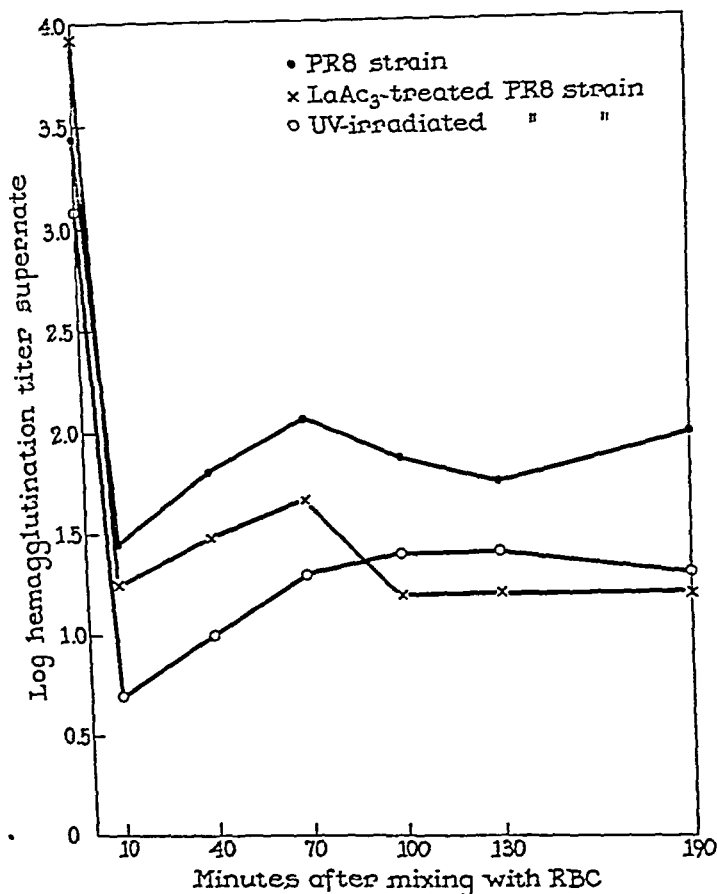


FIG. 2. Stepwise elution curves obtained with the PR8 strain as well as with LaAc_3 -treated and ultraviolet-irradiated strains derived therefrom. The logarithm of the geometric mean of the hemagglutination titers of successive supernates (*cf.* Tables I, II, and IV) is plotted against the time at which the supernate was removed.

contact, *e.g.* 8 hours, with LaAc_3 did not cause any further decrease in the virus infectivity titer.

Elution of Lanthanum-Treated Virus from RBC.—To determine whether treatment with LaAc_3 caused an alteration in the rate of elution of the Lee or PR8 strains was technically difficult with most of the original mixtures because of their markedly reduced hemagglutination titers. However, when a concentration of 0.00025 M was employed with the Lee strain, the hemagglutination

titer was reduced only two- to fourfold and it was possible to demonstrate directly that elution of this treated strain occurred at a definitely slower rate than that of the original strain.

Because virus treated with LaAc_3 retained infectivity for the chick embryo, it was of obvious interest to determine the rate of elution of lanthanum-treated virus from RBC after serial passage in the allantoic sac. A number of experiments were carried out with both the Lee and PR8 strains.

Allantoic fluid pools infected with the desired virus were mixed with LaAc_3 solution so as to yield a concentration of 0.005 M. After 3 to 5 hours at 4°C . and light centrifugation to

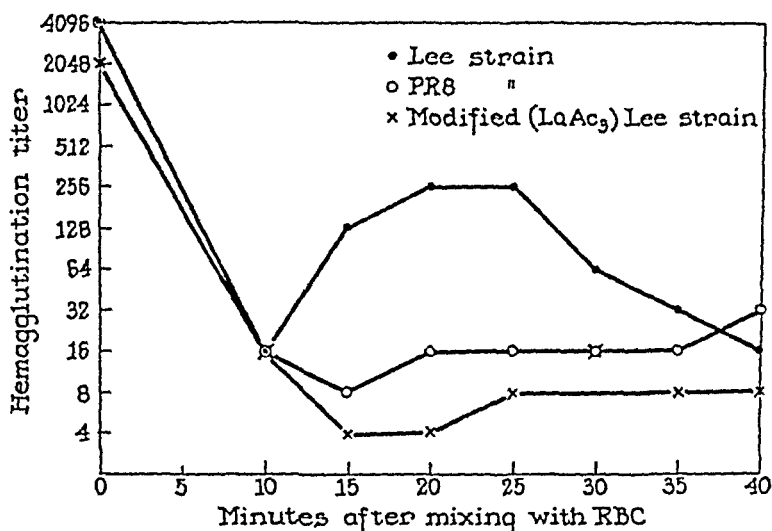


FIG. 3. Stepwise elution curves obtained by removal of the supernate at 5 minute intervals with the Lee and PR8 strains as well as a modified (LaAc_3 -treated) Lee strain.

remove the precipitate, groups of 6 embryos were inoculated intra-allantoically with a 10^{-2} or 10^{-3} dilution of each supernate. The embryos were incubated and their allantoic fluids removed as described above. All fluids from each group which gave positive hemagglutination tests were pooled and used either in elution rate experiments or as inocula for additional groups of embryos. In passages the inoculum was diluted 10^{-3} . No further treatment with LaAc_3 was employed during the serial passage experiments. After a single treatment with LaAc_3 the Lee strain was carried through 15 serial passages in the allantoic sac; the PR8 strain through 7.

The results of stepwise elution rate experiments with LaAc_3 -treated strains of virus after serial passage in the chick embryo are shown in Table II as well as in Figs. 1 and 2. With the Lee strain the allantoic fluid pools employed ranged from the 1st through the 15th serial passage. With the PR8 strain pools obtained at each passage from the 1st through the 7th were used. It

is seen that the rate of elution of the LaAc₃-treated Lee strain from RBC was strikingly different from that of the original Lee strain (*cf.* Table I). The very rapid elution rate during the first 30 minute step was no longer demonstrable and the progressive decrease in the elution rate, which characterized the original strain, was no longer evident. Moreover, the total quantity of virus which was eluted during successive steps was approximately $\frac{1}{3}$ of the

TABLE II

Results of Stepwise Elution Experiments with Lanthanum-Treated Lee and PR8 Strains after Serial Passage in the Chick Embryo

Virus strain (LaAc ₃ -treated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	9	min. 0 (Control)	8192	1024	2352	—
"	"	10	128	4	15	—
"	"	40	512	4	35	1.4
"	"	70	512	8	138	5.6
"	8	100	256	32	70	3.0
"	9	130	128	8	32	1.4
"	"	190	64	8	24	1.1
Total virus eluted.....					299	12.5
PR8	7	0 (Control)	4096	1024	2470	—
"	"	10	64	8	18	—
"	"	40	512	8	24	1.0
"	"	70	256	16	29	1.2
"	"	100	256	16	35	1.5
"	"	130	128	16	39	1.6
"	"	190	128	16	39	1.7
Total virus eluted.....					166	7.0

amount obtained with the original strain. In these respects, the elution of the LaAc₃-treated Lee strain closely resembled that of the original PR8 strain (*cf.* Table I). It seems important to point out that prolonged serial passage with infected allantoic fluid inocula diluted only 10^{-3} , did not cause reversion in the peculiar elution characteristics of the LaAc₃-treated Lee strain; after 15 passages in the absence of lanthanum, the elution curve remained grossly abnormal and was not significantly different from that obtained after but a single passage of the modified strain in the allantoic sac.

It also is seen from the results shown in Table II that the rate of elution of

the LaAc₃-treated PR8 strain from RBC was somewhat slower than that of the original PR8 strain. It should be noted, too, that only about $\frac{1}{2}$ as much of the treated strain as of the original strain was eluted during successive elution steps in the time period studied. Even after 7 serial passages in the allantoic sac in the absence of lanthanum these alterations in the elution curve of the LaAc₃-treated PR8 strain remained demonstrable; no evidence for reversion to the characteristics of the original strain was obtained.

Elution of Ultraviolet-Irradiated Virus from RBC.—Henle and Henle (12) showed that ultraviolet irradiation inactivated various potentialities of influenza virus at different rates. Under the experimental conditions which they employed there was but little effect upon elution of the virus unless irradiation was prolonged sufficiently to affect markedly the capacity of the virus to cause hemagglutination.

In the light of the unexpected and apparently persistent modifications produced by a single treatment with LaAc₃ in the strains under study, it appeared of interest to study further the effects of ultraviolet irradiation upon the Lee and PR8 strains.

Undiluted allantoic fluid pools infected with the desired virus were irradiated with high intensity ultraviolet for varying periods under the conditions described above. After irradiation the hemagglutination titers of the fluids were determined. The longer periods (45 to 60 minutes) of irradiation employed caused a marked reduction in the hemagglutination titer of previously non-irradiated strains. Irradiated fluids were diluted 10^{-3} , and each was inoculated intra-allantoically into a group of 6 embryos. The embryos were incubated and their allantoic fluids harvested as described above. These allantoic fluids were used in elution rate experiments as well as for the inoculation of additional groups of embryos. In passages the inoculum was diluted 10^{-3} . With two irradiated Lee strains 6 serial passages of each, after a single period of irradiation, were carried out in the allantoic sac. With another irradiated Lee strain 11 serial passages were performed and several additional irradiations were carried out between certain passages. With the PR8 strain 7 serial passages were performed and further irradiation was carried out between each passage.

The results of stepwise elution rate experiments with two ultraviolet-irradiated Lee strains after serial passage in the chick embryo are shown in Table III. With these two strains, which were irradiated only once, allantoic fluid obtained after each passage from the 2nd through the 6th was studied. It will be noted that the elution rate of these two irradiated Lee strains was markedly slower than that of the original Lee strain (*cf.* Table I) even though the total amount of virus eluted during all the successive steps was not greatly different. As with the LaAc₃-treated Lee strain (*cf.* Table II), the most striking reduction was demonstrated during the first 30 minute elution period.

The results of similar experiments with a repeatedly irradiated Lee strain are shown in Table IV and in Fig. 1. These experiments were made in an attempt to reduce even more markedly the elution rate of the irradiated Lee strain by means of further irradiation of allantoic fluid between embryo pas-

TABLE III

Results of Stepwise Elution Experiments with Lee Strains after a Single Ultraviolet Irradiation and Serial Passage in the Chick Embryo

Virus strain (ultraviolet- irradiated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	10	min. 0 (Control)	8194	1024	1684	per cent —
"	"	10	256	16	42	—
"	"	40	1024	4	73	4.4
"	"	70	1024	16	157	10.0
"	"	100	256	32	91	6.4
"	"	130	128	8	45	3.4
"	"	190	256	4	42	3.3
Total virus eluted.....					408	27.5

TABLE IV

Results of Stepwise Elution Experiments with Lee and PR8 Strains Repeatedly Ultraviolet-Irradiated after Serial Passage in the Chick Embryo

Virus strain (ultraviolet- irradiated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
Lee	10	min. 0 (Control)	2048	1024	1520	per cent —
"	"	10	128	0	21	—
"	"	40	128	16	32	2.1
"	"	70	512	64	181	12.3
"	"	100	512	32	179	13.9
"	"	130	256	16	60	5.4
"	"	190	128	8	42	4.0
Total virus eluted.....					494	37.7
PR8	7	0 (Control)	4096	1024	2030	—
"	"	10	32	0	5	—
"	"	40	32	0	10	0.5
"	"	70	64	4	20	1.0
"	"	100	128	8	26	1.3
"	"	130	64	8	26	1.3
"	"	190	64	8	20	1.0
Total virus eluted.....					102	5.1

sages. It is evident that the altered elution rate of the irradiated Lee strain was not additionally affected by further irradiation.

It can be seen also from the results shown in Table IV and Fig. 2 that the elution rate of a repeatedly irradiated PR8 strain was somewhat slower than that of the original PR8 strain (*cf.* Table I) and that only approximately $\frac{1}{3}$ as much of the irradiated strain as of the original strain was eluted during the time interval studied. The elution rate of the irradiated PR8 strain corresponded very closely to that of the LaAc₃-treated PR8 strain (*cf.* Table II).

With the irradiated Lee strains no evidence of reversion to the elution characteristics of the original strain was obtained despite serial passage in the embryo with no further irradiation. The elution rates obtained with fluids from the last serial passages, *i.e.* the 6th, appeared not to differ significantly from those obtained with fluids from the 2nd embryo passages. It appears, therefore, that the modifications induced by ultraviolet irradiation, with respect to elution of the strains studied from RBC, persisted on serial passage in the absence of the original agent, as was the case after treatment with LaAc₃.

Infectivity of Modified Strains.—After serial passage in the chick embryo the infectivity titer of LaAc₃-treated Lee and PR8 strains as well as ultraviolet-irradiated Lee and PR8 strains was determined in parallel with that of the original strains. Titrations were carried out both in mice and in chick embryos according to the techniques described above.

In every instance it was found that there was no significant difference between the virus titration end points obtained with the modified and original strains in either mice or chick embryos. It appears, therefore, that both LaAc₃-treated and ultraviolet-irradiated strains retained in undiminished degree the capacity to induce infection in either species.

Immunological Properties of Treated Strains.—After serial passage in the chick embryo cross hemagglutination-inhibition titrations were carried out with LaAc₃-treated, ultraviolet-irradiated, and the original Lee and PR8 strains. The immune sera employed were anti-Lee, anti-PR8, and anti-ultraviolet-irradiated Lee.

In each case it was found that there was no significant difference between the results of quantitative hemagglutination-inhibition titrations with the original strain and with modified strains derived from it. It thus appears that both the LaAc₃-treated and the ultraviolet-irradiated strains retained the immunological specificity which characterizes the strain from which they were derived.

Effect of Increased NaCl Concentration upon Elution Rate.—In this laboratory Davenport (13) recently found that a decrease of electrolyte concentration in RBC-influenza virus systems leads to a reduction in the elution rate of the virus. In the present study, when the NaCl concentration of mixtures was reduced in experiments in which the stepwise elution technique was employed,

a decrease in elution rate also was noted. It was, therefore, of interest to determine what effect an increased salt concentration would have upon the elution rate under similar conditions.

TABLE V

Results of Stepwise Elution Experiments with Lee, PR8, and Ultraviolet-Irradiated Lee Strains in 3 Per Cent NaCl Solution

Virus strain	Time after mixing with RBC	Hemagglutination with successive supernates* diluted as indicated													Total virus eluted in	
		4	8	16	32	64	128	256	512	1024	2048	4096	3.0 per cent NaCl	0.9 per cent NaCl		
	<i>min.</i>															
Lee	0 (Control)	2	2	2	3	3	3	3	3	2	2	2	104	55		
"	10	2	2	3	2											
"	40	0	0	±	2	2	2	2	3	2	2	2				
"	70	3	3	3	3	2	2									
"	100	3	2	2												
"	130	2														
"	190	2														
Ultraviolet-irradiated Lee	0 (Control)	4	4	4	3	3	3	3	3	3	2	2	11	13		
"	10	3	3	2												
"	40	4	3	3	3	2										
"	70	4	4	3	3	3	2									
"	100	4	3	3	3	3	2									
"	130	3	3	3	3	2										
"	190	3	3	3	3	2										
PR8	0 (Control)	4	4	4	3	3	3	3	3	3	2	2	50	10		
"	10	4	3	2												
"	40	4	4	4	4	4	4	3	2							
"	70	4	4	4	4	3	3	2	2							
"	100	4	4	4	4	3	3	2	2							
"	130	4	4	3	3	2	2	2								
"	190	4	4	4	3	3	2	2								

* Dilutions prepared in 0.9 per cent NaCl solution.

A buffered solution containing 3 per cent NaCl, instead of 0.85 per cent, was used. When this solution was added to sedimented RBC and adsorbed virus, it was found that the RBC promptly formed a solid gel. If, however, the RBC were first gradually adapted to an increasing salt concentration by washing successively in 1.5, 2, 2.5, and 3 per cent NaCl solutions, no such difficulty was encountered. The experiment, therefore, was carried out with such NaCl-adapted RBC and 3 per cent buffered NaCl solution was added as an elution medium at each step. The supernates were diluted in 0.9 per cent NaCl in the usual manner.

As can be seen from the results shown in Table V the presence of 3 per cent NaCl in the mixtures increased the elution rate of both the Lee and PR8 strains. However, the modified (ultraviolet-irradiated) Lee strain did not show any significant change in elution rate under these conditions. It should also be noted that in the lower dilutions of the supernate obtained from the Lee strain after the first 30 minute elution step there was no evidence of hemagglutination at 1 hour. With the other supernates from the Lee strain as well as with each of the supernates from the PR8 and the modified Lee strain, the usual results were obtained.

Simultaneous hemagglutination titrations were performed with the Lee and PR8 strains in 3, 2, 0.9, and 0.45 per cent, respectively, buffered NaCl solutions. In 3 per cent NaCl solution the Lee strain causes agglutination rapidly, but after about 25 minutes aggregates disappeared, and at the end of 1 hour only 0 readings were obtained throughout the series of dilutions. In 2 per cent NaCl solution the lowest four dilutions showed 0 readings. No similar disappearance of hemagglutination occurred in either 0.9 or 0.45 per cent NaCl solutions. Identical experiments with the PR8 strain failed to show any such effect of NaCl concentration on the hemagglutination reaction.

It appears that it was possible to increase the elution rate of both the Lee and PR8 strains but not of the modified Lee strain by increasing the NaCl concentration of the elution medium. The disappearance of hemagglutination in the lower dilutions of the supernate from the first 30 minute step is undoubtedly the result of the very rapid elution rate of this fraction which is further accelerated by the increased NaCl concentration. It is of interest that both the PR8 strain and the modified Lee strain, both of which have relatively slow elution rates, failed to show a similar phenomenon.

DISCUSSION

That it is possible to cause certain alterations in a property of influenza virus by well defined and readily controlled laboratory procedures is apparent from the results obtained in this study. A single brief period of treatment with lanthanum acetate in low concentration or a single period of irradiation with ultraviolet light resulted in the development of virus strains which possessed a demonstrable modification as compared with the original strain. Of most interest and importance is the fact that the modified strains retained their unusual character on serial passage in the chick embryo in the absence of the agent which originally produced the alteration.

Insofar as was determined but a single property of the modified strains was altered. That other properties also may have been altered is, of course, possible. However, infectivity for both the mouse and the chick embryo, hemagglutinating capacity, rate and degree of adsorption on RBC, antigenicity and immunological specificity all appeared to be identical with those of the original

strain. Of the properties studied only elution from combination with RBC was demonstrably different, both as to rate and extent, and served to distinguish the modified strains of Lee virus from the original strain.

It is noteworthy that it is the Lee strain which yielded modified strains with the more striking reductions in elution rate. It has been known for some time (2) that the Lee strain elutes from RBC considerably more rapidly than the PR8 strain. The alteration was sufficiently marked to make it impossible to distinguish between modified Lee strains and the PR8 strain on the basis of their rates of elution alone.

As for the agents which were employed for the development of modified influenza virus, it is known that lanthanum forms insoluble complexes with nucleic acids (14) and the wave length of ultraviolet employed appears to be identical with that at which nucleic acids show maximal adsorption (15). Whether either or both agents exert their effects as a result of action on nucleic acid components of the virus particles is hypothetical. However, it appears possible that either agent might deleteriously affect such components.

The fact that the original Lee strain shows a progressively decreasing rate of elution with increasing time suggests that the individual virus particles are not entirely identical as regards this one property. There is, in fact, no good reason for thinking that all virus particles of a given strain are identical in all respects. There is some evidence which strongly suggests that influenza viruses, like numerous other infectious agents, may show variation relative to various properties; *i.e.*, pathogenicity for unnatural hosts (16, 17), capacity to agglutinate erythrocytes from various species (18), and immunological characteristics (19, 20). It seems probable that an infected allantoic fluid can be considered to contain an enormous population of virus particles all fundamentally similar in most respects but differing slightly, one from another, in certain respects. If such an inhomogeneity in a given population of virus particles exists, it would not be surprising that chemical or physical agents might affect individual particles in somewhat different ways. Thus, the deleterious action of lanthanum or of ultraviolet might vary in an inhomogeneous population, and those particles which, following treatment, retained the capacity to multiply in a susceptible host would be in all probability the particles least affected by either agent. Passage of such a treated strain should result in the development of a virus population closely similar to or identical, in distribution of properties, with that of the original strain only if the treated strain were as capable as the original strain of leading to the development of virus particles with slightly diverse properties. If, however, a treated strain contains less than the original distribution of slightly different virus particles, and leads in the susceptible host to the development of a virus population with an abnormally restricted distribution of properties, an altered strain would emerge. Such an altered strain could continue on serial passage to show the same unusual

property, although reversion with the eventual acquisition of the properties of the original strain might occur if passages were continued sufficiently long.

It seems likely that the alteration produced in influenza virus in the present study is best explained on the basis of selection of naturally occurring variants. It is, of course, possible that variants might actually have been induced by the procedures employed. With the modified Lee strains it appears that the relatively large proportion of the virus population of the original strain, which shows a very rapid elution rate, is much reduced. The slower elution rate of the modified strains may be explained by the reproduction in the susceptible host of a higher than normal proportion of virus particles with a slow elution rate due presumably to the selective inactivation of virus particles with a high elution rate by the agents initially employed. Whether variants were selected or induced by the experimental procedures, the final results would be similar and a modified strain could be evolved under either circumstance.

SUMMARY

The rates of elution from RBC of the Lee and PR8 strains of influenza virus were studied by means of a step-wise elution technique. By means of a single treatment with lanthanum acetate or irradiation with ultraviolet and subsequent passage in chick embryos, it was possible to alter the elution rate of the Lee strain so that it was similar to that of the PR8 strain. This alteration proved to be persistent on serial passage in the absence of the agent which caused it. As far as was determined, the elution rate of the virus appeared to be the only property which was altered. The phenomenon can be most readily understood on the assumption that the difference in elution rates of the two strains is due to a heterogeneous population of virus particles in the Lee strain with respect to elution rate.

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REACTIONS BETWEEN INFLUENZA VIRUS AND A COMPONENT OF ALLANTOIC FLUID

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It is well known that there are present in certain tissues of various normal animal species components which are capable of inhibiting hemagglutination by influenza viruses (1-10). Relatively little attention has been given to a component of this kind which is present in the allantoic fluid of normal chick embryos. Svedmyr (7) previously reported upon such a component and studied some of its properties. In view of the fact that, at the present time, most work on influenza viruses is carried out with material obtained from the allantoic sac of the chick embryo, it seemed important to study in detail this inhibitor and the reactions between it and influenza viruses. In the present communication evidence will be presented regarding its nature. Evidence also will be presented to show that it combines with the virus particle, and that following combination the complex dissociates partially, but not completely. In addition, it will be shown that after dissociation the inhibitor is altered, *i.e.* it is no longer capable of combining with additional influenza virus, and therefore cannot be demonstrated directly by the available techniques.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was used. The virus, which previously had been passed many times through chick embryos and mice, was cultivated in the allantoic sac of White Leghorn chick embryos which had been incubated at 39°C. for 10 to 12 days. After inoculation with approximately 10^5 E.I.D. of virus the eggs were incubated at 35°C. for 48 hours, and then chilled at 4°C. overnight before the allantoic fluids were harvested. The infected allantoic fluids employed in hemagglutination experiments were sterile pools obtained from groups of 8 to 10 eggs and were stored at 4°C. Infected fluids which were used for the inoculation of additional eggs were diluted with nine parts of sterile normal horse serum (previously heated at 56°C. for 30 minutes) and stored in nitrocellulose tubes at -70°C.

Normal Allantoic Fluid.—For the most part the normal allantoic fluid used was removed from chick embryos which had been incubated at 39°C. for 14 days. Following incubation the eggs were chilled at 4°C. overnight and the allantoic fluid from groups of 8 to 10 eggs was pooled. Each pool of normal fluid was stored at 4°C. until used. Frequently, a precipitate formed in the allantoic fluid upon storage at 4°C.; this was removed by centrifugation and discarded.

Virus Hemagglutination Titrations.—Serial twofold dilutions of infected allantoic fluid were made in 0.85 per cent NaCl solution buffered at pH 7.2 (0.01 M phosphate). To 0.4 cc. of each dilution in 10 X 75 mm. Pyrex tubes was added 0.4 cc. of a 0.5 per cent suspension of washed chicken RBC in buffered saline. Readings were made after the tubes had stood 1 hour at room temperature. The end point was taken as the highest dilution at which definite (2+) agglutination of the RBC occurred.

EXPERIMENTAL

Increase in the Inhibitor in Extracembryonic Fluids with Age.—Before undertaking a detailed study of the component in normal allantoic or amniotic fluid which inhibits hemagglutination by influenza virus, it was first necessary to determine the age of the embryo at which the fluids contained most inhibitor.

Groups of 10 to 20 normal embryonated eggs were incubated at 39°C. for periods from 7 to 14 days. After incubation the allantoic and amniotic fluids from each group of eggs were pooled separately and each pool was tested for its ability to inhibit hemagglutination with the PR8 strain. Two methods were used: With Method I serial twofold dilutions of a single virus suspension were made in each allantoic or amniotic fluid pool, and the hemagglutination titers obtained were compared with the titer of the virus diluted in buffered saline. With Method II serial twofold dilutions of each pool of allantoic or amniotic fluid were made in buffered saline; to each dilution was added a suspension of heated virus (56°C. for 1 hour) diluted so as to give a final concentration of 4 hemagglutinating units. The titer of the inhibitor was taken as the highest dilution of fluid which prevented definite (1+) hemagglutination.

The results of the experiments with allantoic fluid are presented in Fig. 1 *A*. The curve obtained by Method I is plotted as the difference between the logarithms of the hemagglutination titers obtained when the virus was diluted in normal allantoic fluid as compared to buffered saline. The curve obtained by Method II is plotted as the logarithm of the highest dilution of normal allantoic fluid which prevented hemagglutination by 4 units of virus. It will be seen that both methods gave similar results, although the curve obtained by Method II has a slightly greater slope. The results demonstrate that the concentration of inhibitor in normal allantoic fluid increases progressively with increasing age of the embryo. In general these results are in agreement with the findings of Svedmyr (7) who employed a technique analogous to Method I. However, the present results do not indicate that the concentration of inhibitor is relatively constant after the 11th or 12th day, as was previously suggested (7).

The results of experiments with amniotic fluid are shown in Fig. 1 *B* in which the plotting procedure is identical with that employed in Fig. 1 *A*. Here again, the slope obtained by Method II is greater than that obtained by Method I. It will be noted that there is almost no demonstrable inhibitor in amniotic fluids from normal eggs less than 10 days of age, but that thereafter the concentration of inhibitor increases rapidly with increasing age of the embryo until a peak is reached at 13 days. The inhibitor concentration as determined by Method II at this time is greater than that of allantoic fluid from 14 day embryos. These findings are similar to those of Svedmyr (7) insofar as they may be compared.

Determination of Inhibitor Concentration.—The methods employed to determine the concentration of inhibitor in normal allantoic fluid were of two main types: (a) inhibition of decreasing quantities of virus by a constant quantity

of inhibitor, and (b) inhibition of a constant quantity of virus by decreasing quantities of inhibitor. They are presented in detail below.

Decreasing Virus versus Constant Inhibitor.—In the early experiments an infected allantoic fluid pool was diluted serially in normal allantoic fluid and the hemagglutination titer obtained in the usual manner was compared with the titer obtained after dilution of the same pool in

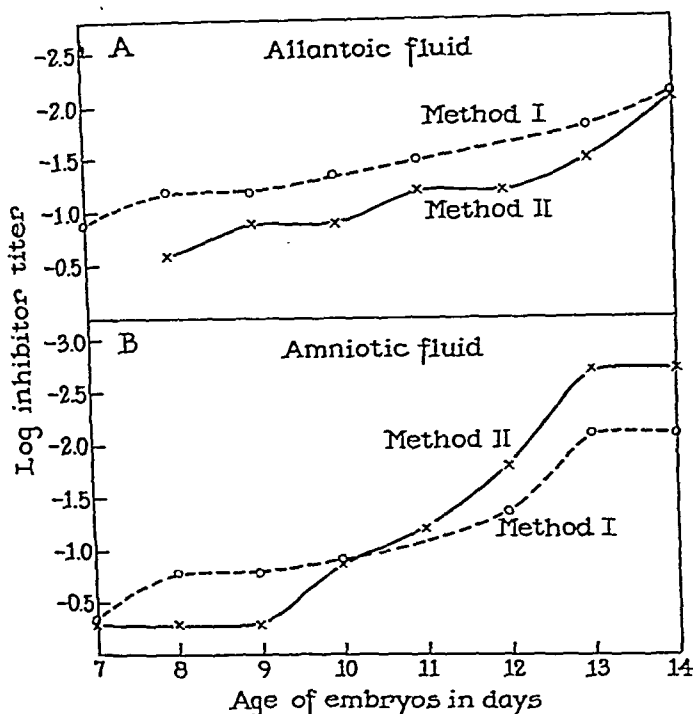


FIG. 1. Increase in inhibitor concentration in extraembryonic fluids with age. Graph A represents results obtained with normal allantoic fluid. Graph B shows the results obtained with normal amniotic fluid. The concentration of inhibitor was determined by two methods: In Method I the virus was diluted in allantoic or amniotic fluid and the log of the difference in titer from a control titration in saline is shown; in Method II dilutions of the fluids were tested against 4 units of heated virus and the log of the highest dilution which inhibited hemagglutination is shown.

buffered saline. In this procedure it is evident that the concentration of normal allantoic fluid increases slightly but progressively with each dilution of the infected pool. In order to control this variable the following procedure was employed.

Serial twofold dilutions of an infected pool were made in buffered saline. To 0.2 cc. of each dilution were added 0.2 cc. of normal allantoic fluid from 14 day old embryos and 0.4 cc. of a 0.5 per cent suspension of washed chicken RBC. The hemagglutination titer obtained was compared with that obtained in a similar titration in which buffered saline was substituted for normal allantoic fluid. The number of hemagglutinating units of virus inhibited was taken as the ratio of the titer in saline to the titer in the presence of normal allantoic fluid.

In Table I the results obtained in a number of experiments carried out by this method are summarized. It will be seen that between 1 and 24 hemagglutinating units of virus were inhibited by normal allantoic fluid when different pools of virus were employed. However, in each instance the quantity of virus inhibited was directly related to the titer of the virus pool used. That is, in the presence of a constant concentration of normal allantoic fluid an almost constant hemagglutination titer is obtained regardless of the level of the original virus titer. Therefore, in order to determine differences in the concentration of inhibitor, it is important to use a virus pool with the highest possible titer.

TABLE I

Relation between Hemagglutination Titer and Quantity of Virus Inhibited by a Constant Amount of Normal Allantoic Fluid

Infected* allantoic fluid pool	Hemagglutination titer†		Virus inhibited
	In buffered saline	In normal allantoic fluid‡	No. of hemagglutinating units
A	256	128	1
B	512	128	2
C	1024	128	4
D	2048	128	8
E	2048	64	16
F	6144	128	24

* Allantoic fluids harvested 48 hours after inoculation of PR8.

† Expressed as the reciprocal.

‡ Obtained from 14 day embryos.

Constant Virus versus Decreasing Inhibitor.—In this procedure serial twofold dilutions of normal allantoic fluid from 14 day embryos were made in buffered saline. To 0.4 cc. of each dilution were added 0.2 cc. of virus, diluted so as to give a final concentration of 4 hemagglutinating units, and 0.2 cc. of a 1 per cent suspension of washed chicken RBC. The titer of the inhibitor was taken as the highest dilution of normal allantoic fluid which prevented definite (1+) hemagglutination.

The results of a typical experiment are shown in Table II. It is seen that normal allantoic fluid inhibited hemagglutination only when it was diluted eightfold or less. Of numerous fluids tested none could be diluted more than 1:8 and still cause inhibition under these conditions.

Constant Heated Virus versus Decreasing Inhibitor.—It was shown by Francis (5) that influenza B virus, when heated at 56°C., lost very little of its hemagglutinating capacity, but gave much higher hemagglutination inhibition titers with normal sera than did equivalent amounts of unheated virus. It seemed possible that similar results might be obtained with normal allantoic fluid, and

consequently titrations were carried out in a manner identical with that described above except that 4 hemagglutinating units of heated virus (56°C. for 1 hour) was used.

The results of a typical experiment with heated virus are also shown in Table II. It will be seen that 4 units of heated virus was inhibited by a much higher dilution of normal allantoic fluid than was an equal amount of unheated virus. In Table III the results of a number of experiments carried out with different

TABLE II

Inhibition Titer of Normal Allantoic Fluid with Infectious and Heated Virus

Infected allantoic fluid		Dilution of normal allantoic fluid*										Inhibition titer
Treatment	Hemagglutinating units per tube	2	4	8	16	32	64	128	256	512	1024	
None	4	0	0	0	1	2	3	4	4	4	4	8
Heated†	4	0	0	0	0	0	0	0	0	2	4	256

* Expressed as the reciprocal.

† 56°C. for 1 hour before dilution.

TABLE III

Relation between Hemagglutination Titer of Virus Pool and Inhibition Titer Obtained with Normal Allantoic Fluid

Infected allantoic fluid pool	Hemagglutination titer	Inhibition titer of normal allantoic fluid*
1	1024	32
2	2048	64
3	2048	128
4	4096	128
5	8192	128
6	8192	256

* Determined with 4 units of virus after heating.

pools of heated virus are summarized. In each instance the titer of inhibitor represents the highest dilution of normal allantoic fluid which inhibited 4 units of heated virus. Because the various virus pools had different titers, the dilution employed varied from one pool to another. It will be seen that 4 units of heated virus, when derived from a high titer pool, was inhibited by a considerably higher dilution of normal allantoic fluid than an equal number of units of heated virus derived from a pool of lower titer. It appears, therefore, that the titer of inhibitor in normal allantoic fluid is influenced by the hemagglutination titer of the virus suspension employed to determine it.

Centrifugation of Inhibitor.—The behavior of the inhibitor in normal allantoic

fluid towards influenza virus suggested that it might be a single component and of moderately large size. This impression was strengthened when it was found not to dialyze through cellophane membranes. To obtain information as to the size of the inhibitor relative to that of influenza virus, the effect of high gravitational fields on the inhibitor and on influenza virus was compared. Svedmyr (7) found that the inhibitor was sedimented at 27,000 R.P.M.

Nitrocellulose tubes containing 20 cc. of either normal allantoic fluid or allantoic fluid from embryos infected with PR8 were centrifuged at 15,000 R.P.M. for varying periods in a high speed vacuum apparatus (11). Both normal and infected fluids were centrifuged simultaneously. The centrifuge head was similar to that previously described (12) but was almost twice as large; the diameter = 30.1 cm. After centrifugation the top 10 cc. of each supernate was removed carefully, the remainder of the supernate was then withdrawn, and the sediment was resuspended in a quantity of buffered saline equal to the original volume. The inhibitor titer of the normal fluid fractions was determined as described above against 4 units of heated virus and the hemagglutination titer of the infected fluid fractions was determined in the usual manner.

The results are presented in Fig. 2 in which the upper portion (A) shows the titers obtained with the top 10 cc. of the supernates and the lower portion (B) shows those obtained with the resuspended sediments. Both the inhibitor and hemagglutination titers are plotted as logarithms. It will be noted that the titer of the inhibitor in the supernates decreased at a much slower rate than did that of the virus and, in addition, that with the resuspended sediments the virus titer increased at a faster rate than did the inhibitor titer. These results indicate that the inhibitor in normal allantoic fluid is significantly smaller than the virus particle (PR8) but show that some sedimentation of the inhibitor is obtained even at a speed of 15,000 R.P.M.

Heat Stability of the Inhibitor.—The effect of heat upon the inhibitor was determined in the following manner:

Specimens of normal allantoic fluid which had been dialyzed overnight against buffered saline (pH 7.2) were heated in a water bath at temperatures of 70 or 100°C. for varying periods. After this treatment the inhibitor concentration of each specimen was determined against 4 units of heated virus as described above. The allantoic fluid, which was almost water-clear before heating, developed only a very slight turbidity even when heated at 100°C. for 1 hour.

The results obtained are shown in Table IV. A twofold decrease in the inhibitor titer occurred after 45 minutes at 70°C. but no further drop in titer was found after 60 minutes. On heating at 100°C. the inhibitor titer decreased somewhat more rapidly but approximately 25 per cent of the inhibitor remained demonstrable even after 1 hour. It is evident that the inhibitor is very stable to heat. Svedmyr (7) showed previously that the inhibitor was not completely destroyed by similar heating.

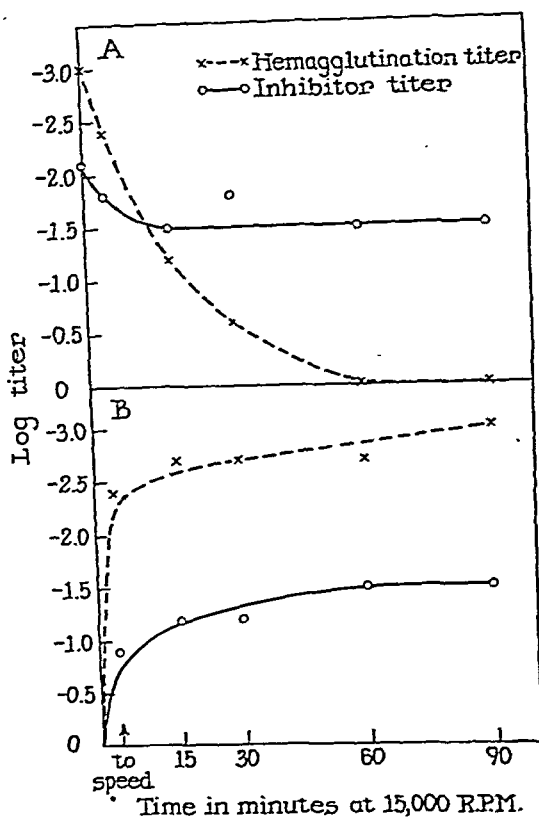


FIG. 2. Effect of high speed centrifugation on the inhibitor as compared with the virus. Aliquots of normal allantoic fluid and infected allantoic fluid were centrifuged simultaneously. Both the hemagglutination titer with infected fluid and the inhibitor titer with normal fluid of the top 10 cc. of the supernates, as shown in Graph A, and of the resuspended sediments, as shown in Graph B, are plotted against the time of centrifugation.

TABLE IV
Effect of Heating on Inhibitor

Treatment of normal allantoic fluid		Inhibition titer*	Treatment of normal allantoic fluid		Inhibition titer*
°C.	min.		°C.	min.	
—	—	128	—	—	128
70	15	128	100	15	128
70	30	128	100	30	64
70	45	64	100	45	64
70	60	64	100	60	32

* Determined against 4 hemagglutination units of heated virus.

Effect of pH on the Inhibitor.—The pH stability range of the inhibitor was determined in the following manner:

Normal allantoic fluid which had been dialyzed overnight against buffered saline was divided into aliquots. The pH of each aliquot was adjusted to the desired level by the drop-wise addition of either 1 N HCl or 1 N NaOH. The specimens adjusted to pH 2.6 and 3.3 became slightly cloudy but no easily sedimentable precipitate formed; the fluid remained clear at the various other pH levels. After 1 hour at room temperature or 30 minutes at 70°C. the inhibitor titer of each specimen was determined against 4 units of heated virus.

The results are summarized in Table V. It will be seen that after 1 hour at room temperature the inhibitor was demonstrable in undiminished concentration at pH levels from 10.7 to 6.3, but at lower pH levels the titer decreased

TABLE V
Stability of Inhibitor at Different pH Levels

Normal allantoic fluid adjusted to ‡	Inhibition titer*	
	After 60 min. at room temperature	After 30 min. at 70°C.
pH		
2.6	16	2
3.3	16	2
4.6	32	16
5.3	32	32
6.3	64	64
7.0	64	64
9.8	64	32
10.0	64	32
10.7	64	16

* Determined against 4 hemagglutination units of heated virus.

‡ With 1 N HCl or 1 N NaOH.

progressively. After heating at 70°C. the inhibitor titer remained constant only at pH 7.0 and 6.3 and was reduced on both the alkaline and acid sides.

Effect of Temperature on Inhibition.—The effect of temperature on the inhibition of influenza virus (PR8) by normal allantoic fluid was studied by the constant inhibitor-decreasing virus technique described above. Identical titrations were carried out simultaneously at 4°C., room temperature (22–26°C.), and 37°C. with the same pools of virus.

The results of several such experiments are summarized in Table VI. The results of a similar experiment carried out with heated virus are also shown. It will be noted that the quantity of virus inhibited at 4°C. was from four- to eightfold greater than the quantity inhibited at room temperature; that the quantities of virus inhibited at room temperature and at 37°C. were almost identical; that when heated virus was employed there was very little difference

between the quantity inhibited at 4°C. and the quantity inhibited at room temperature; and that the quantity of heated virus inhibited at room temperature was the same as the quantity of infectious virus inhibited at 4°C. These results show that the inhibition of hemagglutination by normal allantoic fluid can be increased by lowering the temperature to 4°C., and that the same increase in inhibition can be obtained at room temperature or 4°C. by the use of heated virus.

Action of Enzymes and Periodate on the Inhibitor.—Evidence as to the nature of the inhibitor was sought by studying the effect of different purified enzymes upon its capacity to inhibit hemagglutination. It was found that crystalline trypsin¹ in low concentration failed to alter the inhibitor titer. However, when allantoic fluid which had been dialyzed against buffered saline (pH 7.2)

TABLE VI

Effect of Temperature on Quantity of Virus Inhibited by a Constant Amount of Normal Allantoic Fluid

Infected allantoic fluid pool	Hemagglutination titer*			
	In buffer saline	In normal allantoic fluid		
		4°C.	22-26°C.	37°C.
A	256	32	128	256
B	1024	32	128	128
C	2048	16	128	128
B heated†	1024	16	32	—

* Expressed as the reciprocal.

† 56°C. for 1 hour.

was treated with a relatively high concentration of crystalline trypsin (1.25 mg. per cc.) for 3 hours at 37°C., there was almost complete destruction of inhibitor activity as is shown in Table VII. It is well established that egg white contains a trypsin inhibitor in very high concentration (13), and it seems probable that a similar substance may be present in allantoic fluid. If this were true, it would provide an explanation for the failure of trypsin in low concentration to destroy inhibitor activity.

The effect of crystalline ribonuclease and highly purified desoxyribonuclease on the inhibitor was also determined.

An equal volume of crystalline ribonuclease² (1.0 mg. per cc.) or desoxyribonuclease² (1.0 mg. per cc. in 0.03 M MgSO₄) was added to dialyzed allantoic fluid and the mixtures were

¹ Obtained through the courtesy of Dr. M. Kunitz of The Rockefeller Institute, Princeton, New Jersey.

² These enzymes were kindly provided by Dr. M. McCarty of the Hospital of The Rockefeller Institute, New York.

incubated at 37°C. for 4 hours. Following this the inhibitor titer of each mixture was determined against 4 units of heated PR8.

The results obtained are also shown in Table VII. It will be noted that neither enzyme had any apparent effect upon the inhibitor titer.

Burnet (8) showed that several mucinous substances inhibit hemagglutination by influenza viruses. This prompted the treatment of allantoic fluid with several hyaluronidase preparations.

Four preparations were used: a pneumococcal hyaluronidase³ (containing 1500 viscosity units per mg.) prepared from a type II R (D39R) culture by the method of Meyer *et al.* (14);

TABLE VII
Effect of Enzymes and Periodate on Inhibitor

Treatment of normal allantoic fluid			Inhibitor titer	
Material	Concentration	Time	After treatment	Control not treated
	mg./cc.	hrs.		
Pneumococcal hyaluronidase.....	0.001	1	<2	256
Pneumococcal hyaluronidase (heated)*.....	0.001	1	256	256
Crystalline trypsin.....	1.25	3	4	128
Crystalline ribonuclease.....	0.5	4	128	128
Desoxyribonuclease†.....	0.5	4	128	128
	per cent			
Streptococcal extract.....	10.0	1	64	64
Leech extract.....	10.0	1	64	64
Testicular extract.....	10.0	1	64	64
LiIO ₄	0.025 M	2	<4	64

* 70°C. for 5 minutes in buffered saline.

† In 0.03 M MgSO₄.

a streptococcal extract³ (approximately 100 viscosity units per cc.) prepared from the supernate of a culture of group A, type 4 hemolytic streptococci; a crude bull testicular extract³ (570 viscosity units per cc.) prepared by the method of Kass and Seastone (15); and a leech extract³ (approximately 500 viscosity units per cc.) prepared by the method of Claude (16). The pneumococcal hyaluronidase was dissolved in distilled water and added to dialyzed normal allantoic fluid to yield a concentration of 0.001 mg. per cc.; the streptococcal, testicular, and leech extracts were added to specimens of allantoic fluid to yield a concentration of 10 per cent. The fluids were incubated in a water bath at 37°C. for 1 hour and then at 70°C. for 5 minutes in order to inactivate the various enzymes. After treatment the inhibitor titer of each mixture was determined against 4 units of heated PR8.

The results of these experiments are presented in Table VII. It will be seen that there was no demonstrable inhibitor in allantoic fluid after treatment

³ Kindly provided by Dr. S. Rothbard of the Hospital of The Rockefeller Institute, New York.

with pneumococcal hyaluronidase, whereas the inhibitor titer was undiminished in the fluids treated with streptococcal, testicular, or leech extracts. This raised the possibility that the component of the pneumococcal preparation responsible for the destruction of the inhibitor might be something other than hyaluronidase. The preparation did contain a hemolysin but had no proteolytic activity against gelatin or casein.⁴ The ability of the pneumococcal enzyme to destroy the inhibitor was completely inactivated after heating for 5 minutes at 70°C. in the presence of allantoic fluid, buffered saline, or phosphate buffer (pH 7.1, μ 0.5); the hyaluronidase activity as determined by the viscosimetric method was also destroyed under these conditions.⁴

Meyer *et al.* (17, 18) compared hyaluronidases of different origin by both viscosimetric and reductometric methods and found that pneumococcal hyaluronidase hydrolyzed the substrate to almost 100 per cent of the theoretical amount, but that testicular and leech hyaluronidases, both of which contained more activity than the pneumococcal preparations in viscosimetric tests, hydrolyzed the substrate to only 50 and 40 per cent, respectively. They concluded that hyaluronidases were mixtures of at least two enzymes, one attacking the long chain molecules, the other hydrolyzing the aldobionic acid units. This could provide an explanation for the failure of the streptococcal, testicular, and leech extracts to destroy inhibitor activity. The very low concentration of pneumococcal hyaluronidase required to destroy inhibitor activity supports the idea that one of the hyaluronidase enzymes was responsible for the destruction of inhibitor activity.

Hirst (10) showed that sodium periodate destroys the virus receptors of red blood cells and also the hemagglutination inhibitor in normal rabbit serum. He suggested that this effect was due to the alteration of a polysaccharide by periodate. The effect of periodate on the inhibitor in allantoic fluid was therefore studied.

One volume of 0.1 M $LiIO_4$ was added to 3 volumes of normal allantoic fluid. After 2 hours at room temperature 2 volumes of 7.5 per cent glucose was added to inactivate the remaining periodate, and the inhibitor titer was then determined against 4 units of heated PR8.

The results of this experiment are shown in Table VII. It will be seen that insofar as could be determined the inhibitor was completely inactivated by lithium periodate.

It appears that three substances have been found, each of which is capable of inactivating the inhibitor in normal allantoic fluid. These are: crystalline trypsin, pneumococcal hyaluronidase, and lithium periodate. The destruction of inhibitor activity by crystalline trypsin suggests that at least part of the inhibitor is protein, while inactivation by pneumococcal hyaluronidase as well as periodate suggests that the inhibitor contains carbohydrate. The available

⁴ These determinations were kindly carried out by Dr. M. McCarty of the Hospital of The Rockefeller Institute, New York.

evidence is consistent with the idea that the inhibitor is, in all probability, a mucoprotein.

Combination between Inhibitor and Virus.—It seemed possible that the inhibitor might prevent hemagglutination in either one of two ways: it could block the virus receptors on the RBC, or it could combine with the virus itself. In order to test the first possibility, chicken RBC were suspended in normal allantoic fluid for varying periods, then sedimented and resuspended in buffered saline. Such treated RBC were found to be agglutinated by both infectious and heated virus equally as well as untreated RBC. These results indicate that the inhibitor did not combine with virus receptors on the RBC. In order to determine whether the inhibitor combined with the virus itself, the following experiment was carried out.

Serial twofold dilutions of normal allantoic fluid were made in duplicate in buffered saline. To each dilution were added 4 units of infectious PR8 and sufficient RBC to yield a concentration of 0.25 per cent. To similar duplicate dilutions of normal allantoic fluid were added 4 units of heated virus and 0.25 per cent RBC. The inhibitor titer with one series of dilutions containing infectious virus and one containing heated virus was determined in the usual manner. In the case of the other two series the RBC were sedimented immediately after mixing by centrifugation at 2,500 R.P.M. for 2 minutes, the supernate from each tube was discarded, and the cells were resuspended in a quantity of buffered saline equal to the original volume. In every instance the inhibitor titer was determined after 1 hour at room temperature.

The results obtained from this experiment are shown in Table VIII. It will be noted that the inhibitor titer determined against 4 units of infectious virus in the usual manner was very low but that with the duplicate titration, in which the RBC were sedimented immediately and resuspended in saline, the apparent inhibitor titer was 16-fold higher. This indicates that the virus was not sedimented with the RBC in those tubes which contained sufficient inhibitor. When heated virus was used, the inhibitor titer was high in the case of both titrations. The results of this experiment suggest that the inhibitor combines with the virus itself and thereby prevents adsorption of the virus by RBC; that infectious virus is eluted rapidly although incompletely from the inhibitor and is then capable of uniting with RBC; and that heated virus is almost completely incapable of dissociating from the inhibitor just as it is incapable of dissociating from RBC (19). It seems apparent that once the virus-inhibitor reaction has reached equilibrium RBC are incapable of causing the virus to dissociate from inhibitor. If this were not the case, inhibition of hemagglutination in the presence of normal allantoic fluid would not be demonstrable.

The Rate of the Reaction between Virus and Inhibitor.—It was suggested by the results obtained in the preceding experiment that influenza virus combined with inhibitor and then rapidly although incompletely dissociated from it. In order to obtain additional information concerning the reaction between virus and inhibitor, the following experiment was carried out.

Serial twofold dilutions from 1:2 to 1:16 of virus were made in undiluted normal allantoic fluid and immediately placed in a water bath at 37°C. Aliquots of each dilution mixture were removed after varying times and immediately heated at 56°C. for 1 hour in order to stop the reaction between virus and inhibitor. The hemagglutination titer of each aliquot was then determined in the usual manner. The inhibitor titer of each aliquot against 4 units of heated virus was also determined after the aliquot had been heated additionally at 70°C. for 5 minutes in order to completely eliminate the hemagglutinating activity of the virus originally present in it.

The results of this experiment are presented graphically in Fig. 3 in which the upper portion (A) shows the virus hemagglutination titers and the lower

TABLE VIII
Evidence for Combination between Inhibitor and Virus

Mixture				Centrifugation*	Dilution of normal allantoic fluid												Inhibitor titer
PR8-infected allantoic fluid		Normal allantoic fluid	RBC		2	4	8	16	32	64	128	256	512	1024	2048		
Treatment	Hemagglutinating units per tube				per cent	R.P.M.											
None	4	Indicated dilution	0.25	None	0	0	0	1	2	3	4	4	4	4	4	8	
	4	Indicated dilution	0.25	2500	0	0	0	0	0	0	0	1	4	4	4	128	
Heated†	4	Indicated dilution	0.25	None	0	0	0	0	0	0	0	0	2	4	4	256	
	4	Indicated dilution	0.25	2500	0	0	0	0	0	0	0	0	0	2	4	512	

* Mixture centrifuged immediately after preparation. Sedimented RBC resuspended to volume in buffered saline.

† Undiluted fluid heated at 56°C. for 1 hour.

portion (B) the inhibitor titers plotted as logarithms against the time of incubation at 37°C. It will be noted that, when the mixtures of virus in normal allantoic fluid were heated at 56°C. for 1 hour immediately after preparation, hemagglutination was demonstrable only in the mixture with the lowest dilution of virus, *i.e.* 1:2, and then only in very low titer. However, when the mixtures were incubated at 37°C. for varying times before heating at 56°C., the hemagglutination titer of each mixture increased progressively with time. The rate of increase in titer became slower as the ratio of virus to normal allantoic fluid decreased, and the maximum titer reached with each mixture was progressively lower than would have been expected in terms of the quantity of virus present in the mixture. The inhibitor titers determined with the same

mixtures showed that the quantity of demonstrable inhibitor decreased progressively as the hemagglutination titer increased, and the inhibitor completely disappeared from each mixture at the same time that the hemagglutination titer reached a maximum level except with the mixture which originally con-

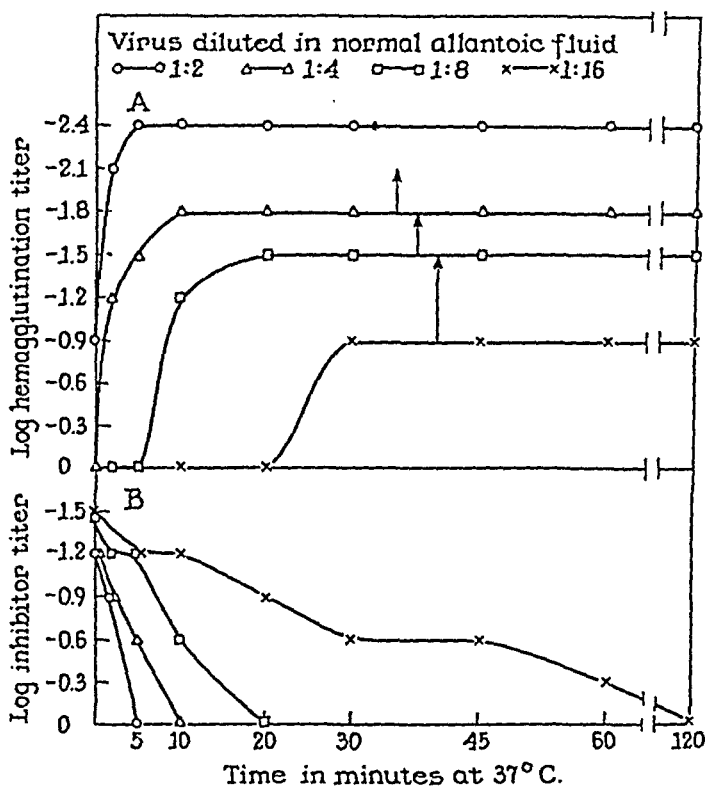


FIG. 3. The rate of the reaction between virus and inhibitor. Serial twofold dilutions of virus in allantoic fluid were incubated at 37°C. Aliquots were removed after varying periods and immediately heated at 56°C. for 1 hour. The hemagglutination titer of each aliquot is shown in Graph A. The arrows above each curve indicate the level which the hemagglutination titer should have reached if all virus had been released from combination with inhibitor. The inhibitor titer against 4 units of heated virus, after each aliquot had been heated additionally at 70°C. for 5 minutes, is shown in Graph B.

tained a 1:16 dilution of virus. In this mixture there was still a small amount of demonstrable inhibitor present when the hemagglutination titer reached a maximum level, but even in this instance the inhibitor titer decreased on further incubation. These findings provide strong evidence that the virus combines with the inhibitor and then dissociates partially from it. They also indicate that combination between the virus and the inhibitor takes place very rapidly,

and that upon completion of the reaction the inhibitor is no longer demonstrable. Furthermore, the results of this experiment indicate that the quantity of virus which is capable of dissociating from inhibitor is dependent upon the ratio of virus to inhibitor; the lower the ratio, the less virus is released.

Quantitative Relation between Free and Combined Virus.—Additional information concerning the effect of the virus-inhibitor ratio on the quantity of virus capable of remaining combined with inhibitor was obtained in the following manner:

Serial twofold dilutions of infectious virus were made in buffered saline and to each dilution was added an equal quantity of normal allantoic fluid. Thus, the inhibitor concentration in the resulting mixtures remained constant while the virus concentration progressively decreased. The mixtures were held at room temperature for 30 minutes or longer so as to reach equilibrium. In certain experiments the mixtures were held at 37°C. for as long as 24 hours. The hemagglutination titer of each mixture then was determined in the usual manner. The quantity of virus combined with inhibitor, *i.e.* the quantity of virus incapable of dissociating from inhibitor, in each mixture was taken as the difference between the total quantity of virus added to the mixture and the quantity of free virus demonstrable by hemagglutination.

The results obtained in three separate experiments are plotted graphically in Fig. 4: curve I was fitted to points which show the logarithm of the quantity of virus combined with inhibitor plotted against the logarithm of the total quantity of virus added; curve II was fitted to points which show the logarithm of the quantity of free virus plotted against the logarithm of the total quantity of virus added. It will be noted that as the total amount of virus in the mixtures decreased, *i.e.* as the ratio of virus to inhibitor decreased, relatively less free virus was demonstrable, and a higher proportion of the total virus remained combined with inhibitor. Under these circumstances it would be expected that curves I and II should diverge from each other as, it will be seen, they do. Thus it appears that in the presence of a constant concentration of inhibitor the quantity of virus which remains combined at equilibrium is a function of the total quantity of virus present in the mixture. In this connection it is of importance to recall that with infected allantoic fluids which showed various hemagglutination titer levels, *i.e.* free virus, the quantity of virus which combined with a constant amount of added inhibitor was directly related to the free virus level (*cf.* Table II). It appears that the reaction between influenza virus and inhibitor can be expressed in terms of the usual equilibrium equation:



in which VI represents virus (V) combined with inhibitor (I). It should, however, be emphasized that the inhibitor is altered by the virus as is the case also with the inhibitor present in normal serum (19). It was found that incubation of mixtures of virus and inhibitor at 37°C. for periods as long as 24 hours did not lead to any consistent alteration in the equilibrium; no additional

free virus was released from combination after the first few minutes of the reaction.

Inhibitor in Egg White.—Numerous attempts were made to concentrate and purify the inhibitor in normal allantoic fluid by a number of procedures with

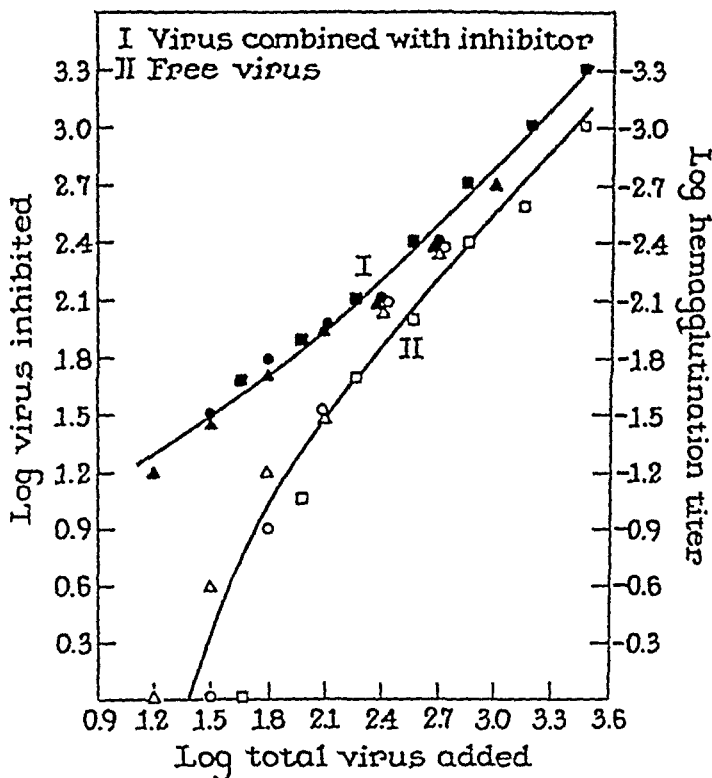


FIG. 4. The equilibrium between virus and inhibitor. Equal portions of undiluted allantoic fluid were added to serial twofold dilutions of virus. After 30 minutes at room temperature the quantity of free virus in each mixture was determined by the hemagglutination technique. The quantity of virus combined with inhibitor, curve I, was determined from the difference between the total quantity of virus added and the quantity of free virus found, curve II.

very little success. This was partially attributable to the very low concentration of the inhibitor and therefore a richer source derived from the egg was sought. It was found that egg white in very high dilution was capable of inhibiting hemagglutination: a 0.25 per cent solution of egg white in saline had approximately the same inhibitor activity as undiluted normal allantoic fluid. The inhibitor in egg white was found to react with influenza virus in the same manner as does that in allantoic fluid, and its properties, *i.e.* heat stability, pH stability, sedimentation in the centrifuge, and inactivation by enzymes, were

closely similar to those of the inhibitor in allantoic fluid. Therefore, it appeared that the two substances were very closely related if not identical.

Purification of the inhibitor present in egg white was undertaken.

Ovalbumin, ovomucoid A (ovomucin), and lysozyme, substances fairly readily separated from egg white, were found to have little or no inhibitor activity. However, it was found that most of the inhibitor could be sedimented with the precipitate which formed when egg white was diluted with 3 to 4 volumes of distilled water, or when a solution of egg white in 1 per cent saline was brought to 0.3 saturation with ammonium sulfate. Slight purification could be obtained with either method by redissolving the precipitate in 1 per cent saline adjusted to pH 8.0 and reprecipitating several times as before. However, with each additional precipitation the material became increasingly more insoluble.

The most highly purified preparation of the inhibitor was obtained when egg white was fractionated by a cold-alcohol precipitation method adapted from that devised by Cohn *et al.* (20) for fractionating serum proteins. By this method most of the inhibitor activity was found in the fraction which was precipitated at -3°C ., pH 7.9, and an ethanol concentration of 25 per cent. This fraction was extremely difficult to put into solution from the dried state even with violent stirring. It was found that the final material in a concentration of about 0.06 mg. per cc. in 1 per cent saline inhibited the same quantity of heated virus as does normal allantoic fluid. On this basis it can be computed that 1 unit of heated virus should be inhibited by approximately $0.5\text{ }\mu\text{g}$. of the purified material. Chemical analyses on this fraction were not made but it was found to give both a positive Molisch reaction for carbohydrate and a positive biuret reaction for protein.

Inhibitor in Infected Allantoic Fluid.—On numerous occasions attempts were made to demonstrate the presence of inhibitor in infected allantoic fluid after the virus had been either inactivated or removed. The virus was inactivated by either heat or treatment with alkaline pH; *i.e.*, 10 or more; it was removed from the allantoic fluid by either centrifugation or cold-alcohol precipitation. On no occasion was it possible to demonstrate any inhibitor activity whatsoever in infected fluid. In view of the results of the various experiments described above, it would not be expected that inhibitor could be demonstrated in infected allantoic fluid. Because of the prolonged opportunity for contact with virus during the course of multiplication, it would be anticipated that some of the inhibitor should be combined with virus while the remainder, although dissociated from combination with virus, should be altered as a result of viral action. In neither instance could present methods demonstrate the presence of the component.

Bound Virus in Infected Allantoic Fluid.—It is evident from the results of preceding experiments that virus bound to inhibitor is unable to agglutinate RBC. There is, however, no evidence that bound virus is incapable of adsorption to RBC. Nonetheless, it seemed possible that, if part of the virus in

infected allantoic fluid actually were bound to inhibitor, the free virus in the fluid might be separated from the bound virus by adsorption onto and elution from RBC. If such a separation were accomplished, then free virus alone should react differently with added fresh inhibitor than the original infected fluid, *i.e.* more free virus should be bound by fresh inhibitor in the absence of bound virus than in its presence because, as is shown above, the total quantity of virus which combines with inhibitor is directly related to the ratio of virus to inhibitor. However, it was found that virus which had been adsorbed onto and eluted from RBC reacted with fresh inhibitor in a manner quantitatively identical to that of the original infected fluid. It would appear, therefore, that adsorption onto and elution from RBC does not provide evidence of a separation of free from bound virus.

It was found also that there was no significant reduction in the embryo infectivity titer of virus which had been serially diluted in normal allantoic fluid so as to provide a great excess of inhibitor; the 50 per cent embryo infectivity titer was $10^{-5.0}$, whereas that of the control was $10^{-8.5}$. This indicates that virus bound to inhibitor is equally as infectious for the embryo as is free virus. It has been shown (21) that when influenza virus is adsorbed onto RBC from infected allantoic fluid, the extent of the decrease in the hemagglutination titer is closely paralleled by a decrease in the infectivity titer. This evidence suggests that bound virus is adsorbed onto RBC to an extent similar to that of free virus. If this were not the case, the hemagglutination titer, which is a measure of free virus, should be decreased by RBC adsorption to a greater extent than the infectivity titer.

DISCUSSION

It appears evident from the results obtained in this study that there is in normal allantoic fluid a component which is capable of combining with influenza virus and that virus which is combined with the component is incapable of causing hemagglutination. When normal allantoic fluid is added to infected allantoic fluid, the inhibiting component present in the former fluid promptly combines with the free virus present in the latter fluid. This reaction reaches equilibrium in a short period of time and the relative proportions of the components which enter into it then remain constant so long as their concentrations are not changed by the addition of more inhibitor or more virus. Present evidence indicates clearly that although some of the virus dissociates from the inhibiting component after combination not all of the virus is released from combination and a relatively large proportion remains in stable union with the component. As a result of experiments in which the concentrations of virus and inhibitor were varied with respect to each other, it was found that the quantity of virus which remains combined with a constant amount of inhibitor and obviously also the quantity which dissociates from inhibitor are

directly related although not strictly proportional to the quantity of virus present in the system.

It is apparent that there is a striking difference between the virus-inhibitor reaction and the virus-erythrocyte reaction. The latter reaction appears to proceed to completion and all or almost all the virus dissociates from RBC (21). The former reaction appears not to go to completion but instead reaches a fixed equilibrium. Over the range of variables studied the smallest proportion of virus which remained combined with inhibitor was at least 50 per cent and the largest 100 per cent. Following partial dissociation of the virus-inhibitor complexes, the inhibitor which is released is so altered that it can no longer be demonstrated directly by available techniques. In this respect, the reaction is analogous to the virus-erythrocyte reaction. It is well established that on dissociation of the latter combination the RBC also are altered and do not again combine with the virus (21). It follows directly from these considerations that whatever quantity of the inhibiting component may be free in infected allantoic fluid would be expected to be similarly altered and not demonstrable as a consequence. It will be recalled that all attempts to demonstrate the presence of unaltered inhibitor in infected fluid were unsuccessful. It is, of course, possible that infected fluid is actually devoid of the inhibiting component and that all which was present at the time infection was initiated was not only altered as a result of contact with the virus but actually was destroyed or otherwise removed from the allantoic fluid during the course of infection. This seems a forced and unlikely assumption, particularly because there is evidence which indicates that virus combined with inhibitor is present in infected fluid. In the light of the quantitative relationship between inhibitor concentration and virus concentration it can be shown that, if inhibitor remains present and in constant concentration in infected allantoic fluid, proportionately more virus should be combined in fluids of low virus concentration than in fluids of high virus concentration. Therefore, the addition of fresh inhibitor to infected fluids should lead to the binding, *i.e.* inhibition, of less free virus in fluids of low virus concentration than in fluids of high virus concentration. It was found that such a relationship can be demonstrated.

The evidence obtained in this study makes it appear highly probable that allantoic fluid infected with influenza virus contains at least three components which are in equilibrium: (1) free virus (which is capable of causing hemagglutination), (2) altered inhibitor (which is no longer capable of combining with virus), and (3) virus combined with inhibitor. Under these circumstances the addition of normal allantoic fluid to infected allantoic fluid serves merely to upset an existing equilibrium which then promptly becomes reestablished at a different level. The increased inhibitor concentration of the system should lead to an increase in the amount of bound virus at the expense of free virus. The evidence indicates that this occurs. It is important to point out that virus

combined with inhibitor appears to be equally as infectious for the chick embryo as free virus.

Many of the characteristics of the hemagglutination reaction with influenza virus suggest that erythrocytes are agglutinated as a direct result of their combination with virus particles and not because of secondary alterations produced in the red blood cells. Indeed, Heinmets (22) in studies on the RBC-virus reaction with the electron microscope obtained evidence which suggests that virus particles form linkages between erythrocytes and thereby cause them to agglutinate. There appear to be adequate reasons for thinking that the virus is "divalent" or possibly "multivalent" with respect to red blood cells; *i.e.*, a single virus particle can combine with more than one erythrocyte simultaneously. If it is assumed for purposes of simplification that the virus is merely divalent, *i.e.* can combine with but two erythrocytes simultaneously, then it is probable that both "valences" should be free or uncombined if hemagglutination is to occur. In terms of this hypothesis, if one valence were combined with inhibitor while the other remained free, hemagglutination should not occur even though the virus should still be capable of adsorption onto and elution from RBC. It seems likely that such a situation may actually exist in infected allantoic fluid and that some of the virus which is combined with inhibitor can also unite with erythrocytes. That RBC can adsorb virus particles without becoming agglutinated has been shown recently (22). In view of these considerations it would appear that present procedures for the purification of influenza viruses probably do not permit of a complete separation of virus particles from combined inhibitor. In this connection, it is of interest that Cohen (23) found that highly purified influenza virus preparations obtained from infected allantoic fluid contained a considerable amount of antigenic material characteristic of the host. Knight (24) obtained similar results with highly purified preparations from both allantoic fluid and mouse lung suspensions and expressed the opinion that host material was incorporated into the virus particle itself. The results of the present study suggest that the host component, *i.e.* inhibitor, is bound to the surface of the virus particle as a result of a definite reaction and is, in all probability, not an essential constituent of the virus.

On the basis of chemical analyses Knight (25) concluded that the virus particles contain a polysaccharide in addition to other substances. He also found an appreciable amount of glucosamine in sedimentable material from normal allantoic fluid and suggested that the amount of host material in purified virus-containing particles could be ascertained from their glucosamine content. Inasmuch as the results obtained in the present study indicate that the inhibitor present in allantoic fluid is probably a mucoprotein which is capable of forming a stable combination with the virus, it would appear that a simple

explanation may be offered for some of the results previously obtained in immunological and chemical studies on purified preparations.

The presence of virus-inhibitor complexes in infected allantoic fluid may have consequences of both practical and theoretical significance. Mention has been made of the difficulties of separating free virus from bound virus either in the centrifuge or by adsorption and elution from RBC. Because hemagglutination titrations serve to measure free virus, but not virus bound by inhibitor, they may not reflect accurately the total concentration of virus in an infected allantoic fluid. Moreover, if different strains of virus react in quantitatively different manners with inhibitor, an inconstant ratio between infectivity and hemagglutination titers might be anticipated. It is well known that freshly recovered strains may give very peculiar and irregular results both in hemagglutination and in hemagglutination-inhibition titrations in the presence of immune serum. It seems probable that certain of the unusual reactions obtained with such strains may be attributable to the presence of virus-inhibitor complexes.

SUMMARY

Evidence is presented which shows that there is a component present in normal allantoic fluid, probably mucoprotein in nature, capable of combining with influenza A virus (PR8), and that following combination between this component and the virus only partial dissociation of the complex occurs. Evidence is also presented which strongly suggests that the component is present in virus-infected allantoic fluid in which it is in part combined with the virus and in part free although altered by viral action. The probability that the component is present as well in highly purified preparations of influenza virus, and its effect upon various reactions obtained with this agent are discussed.

Addendum.—In a recent paper Lanni and Beard⁵ reported that egg white is highly effective in inhibiting hemagglutination by heated swine influenza virus and suggested that this capacity is attributable to a component which combines with the virus.

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THE RÔLE OF THE "WAX" OF THE TUBERCLE BACILLUS IN ESTABLISHING DELAYED HYPERSENSITIVITY

I. HYPERSENSITIVITY TO A SIMPLE CHEMICAL SUBSTANCE, PICRYL CHLORIDE*

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PLATE 23

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One highly puzzling aspect of the phenomenon of the delayed hypersensitivity of infection, namely, the necessity for the presence of the entire organism in the tissues in order that the hypersensitive state be induced, has been dealt with in previous publications in relation to tuberculosis (1, 2). Although it is well known in the case of the tubercle bacillus that the protein of the organism is the antigenic component responsible for the hypersensitive state, this substance in isolated form is powerless to bring about the typical hypersensitivity which follows either infection or the injection of killed bacterial cells, and this despite the well established antigenic properties of such protein. It was demonstrated that the protein could become effective if at the same time the animal received another component of the bacillus; *i.e.*, the waxy lipid extractable with chloroform (3). A series of criteria established that the hypersensitive state so induced fulfills in all particulars that which follows on the heels of tuberculous infection.

In work of this nature, a point of confusion may arise from the failure to distinguish—in the mind of the investigator as well as in the inductive procedures employed—the nature of delayed hypersensitivity and the basic differences segregating it from hypersensitivity of the immediate type, including anaphylaxis, Arthus reactivity, and the human "atopic" states. Briefly stated, the differences are these:

1. Immediate hypersensitive reactions follow exposure to antigen within seconds or minutes. Delayed reactions require hours and progress relatively slowly.

2. In immediate hypersensitivity a demonstrable humoral antibody is involved, since this will transfer the state to normal recipients. In delayed reactivity no antibody has ever been demonstrated; the only successful attempts to transfer the state have been those employing cells obtained, for example, from an induced peritoneal exudate (4-8).

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3. In immediate hypersensitivity certain types of tissue, preeminently smooth muscle and vascular endothelium, are susceptible to the effects of antigen-antibody union. This susceptibility is revealed by contraction in the first instance, and by increased vascular permeability or even damage extending to thrombosis and rupture of vessels in the second. In delayed hypersensitivity all varieties of cells become directly susceptible to the action of antigen, presumably because of their content of some immunologically induced change analogous to antibody, although the nature of this change has never been directly revealed. Such cells may undergo damage extending to necrosis on contact with antigen even when growing in culture in a medium containing no antibody.

As a consequence of the observations on tuberculous hypersensitivity, thoughts of a more general nature suggested themselves, and with one we are concerned here. If a particular lipid of the tubercle bacillus can exert its effect with an antigen—the tuberculo-protein—from the same organism, it might also cause a similar effect with other, non-related antigens. The present work comprised such a study with a simple antigenic substance, picryl chloride.

Picryl chloride was chosen because it serves as a good example of a simple chemical hapten capable of spontaneous combination with protein and conversion to complete antigenic form after injection into the tissues (9-11). In addition, Landsteiner and Chase (12, 13), Gell and coworkers (11), and Chase (14) have provided an extensive immunological characterization of this substance, including the serological and hypersensitive manifestations to it under different conditions. The immunological facts established have been these: that picryl chloride is antigenic when administered by any parenteral route, inducing antibodies and anaphylactic (immediate) hypersensitivity. If, however, it gains entrance into the body via the *skin*, either by applications to the surface or by intracutaneous injection, there is established in addition a state of delayed reactivity to the substance so that future application to the skin results in a typical contact dermatitis, developing slowly and with no resemblance to an Arthus phenomenon. This state has been shown to be independent of the simultaneous existence of humoral antibodies and anaphylactic reactivity, for Landsteiner and Chase (12) have desensitized to the latter and demonstrated that the delayed contact reactivity remains. It is essential for the induction of the contact reactivity that the drug gain access to the body by way of the skin.

Now this description of events under ordinary conditions of sensitization with picryl chloride becomes altered when special circumstances are introduced. Thus, Landsteiner and Chase (15) showed several years ago that if killed tubercle bacilli were injected along with picryl chloride in an oil menstruum *intra-peritoneally*, guinea pigs developed delayed reactivity to the chemical. This work was in fact a specialized extension of earlier observations concerning

a change in the type of hypersensitive response following injection of various antigenic substances into tuberculous lesions (Dienes and Schoenheit (16-19), Hanks (20)), as well as lesions induced with killed tubercle bacilli (19, 21). Later similar observations were made with killed bacilli mixed with antigens (19, 21-26).

A summary of these various observations and their mutual relationships is in essence as follows:—

(a) The isolated protein antigen of the tubercle bacillus cannot induce tuberculin hypersensitivity as can the entire organism.

(b) The wax component of the bacillus when administered with the protein causes the delayed tuberculin response.

(c) Picryl chloride induces delayed hypersensitivity only when administered through the skin, but if tubercle bacilli are injected along with picryl chloride by another route (intraperitoneally) a delayed hypersensitive responsiveness to the chemical ensues.

From these facts the consequence appears probable that the wax of the tubercle bacillus may determine the delayed type of response to picryl chloride equally as well as it determines this kind of response to tuberculo-protein. Such a demonstration may provide the beginning of a chemical basis for generalization concerning the factors determining the occurrence of delayed hypersensitivity.

EXPERIMENTAL

Picryl chloride (Eastman Kodak Company) was purified by several recrystallizations from a benzene-alcohol mixture. Picrylated serum antigen was prepared from guinea pig serum by the method of Landsteiner and Chase (12). Purified wax of the tubercle bacillus was obtained from H37Rv organisms, employing the chemical procedures of Anderson (3). The repeated ultrafiltrations and ultracentrifugations carried out in order to free the lipid of residual bacillary bodies are described in detail in a preceding report (2).

Preparations of Picryl Chloride for Injections.—When injections were made in saline as the vehicle, a solution of 15 mg. of picryl chloride per ml. in absolute alcohol was added drop by drop to the saline to a final concentration of 0.5 mg. per ml.

For intracutaneous injections, 0.10 ml. of a solution containing 0.0025 mg. of picryl chloride was employed.

For subcutaneous injections, 0.4 ml. containing 0.20 mg. of picryl chloride was used.

For intraperitoneal injections, 1.0 ml. containing 0.50 mg. of picryl chloride was used.

Picryl chloride in water-in-oil emulsion was prepared by the method of Freund (22). To 0.4 ml. of melted aquaphor, 0.4 ml. of warm saline was added drop by drop, mixing continuously with a pestle. To this mixture was added 4.0 mg. of solid picryl chloride. Then 1.2 ml. of warm paraffin oil was mixed in and the whole thoroughly emulsified in a mortar. The intraperitoneal dose consisted of 0.25 ml. of emulsion containing 0.5 mg. of picryl chloride.

For skin applications, a 1.5 per cent solution of picryl chloride in olive oil was employed. One drop was applied to the shaved skin and spread over an area of about 2.5 cm. with a glass rod. Each application thus consisted of about 1.0 mg. of the chemical.

The intraperitoneal injection of picryl chloride and wax was carried out as follows:—5 mg. of wax in emulsion in 0.5 ml. of distilled water was first injected. Following this, an injection was made of 0.5 mg. of picryl chloride in 1.0 ml. of saline.

In all injections made beyond the integument, precautions were observed to guard against contamination of the skin by the picryl chloride (15). A 1.0 cm. slit was made in the skin of the flank. The skin edges were retracted, and the picryl chloride preparation was introduced subcutaneously or intraperitoneally through a clean 26 gauge needle. After withdrawal, the wound was blotted with petroleum ether followed by alcohol, and 10 per cent thymol iodide in paraffin oil was applied.

Cutaneous Tests.—Contact tests were carried out by application to the shaved skin of the flank or the abdomen of the same 1.5 per cent solution of picryl chloride in olive oil used for the sensitizing inunctions.

Intracutaneous testing consisted of the injection of 0.05 mg. of picryl chloride in 0.1 ml. of saline. This substance induced some irritation, but not sufficient to interfere with readings. Later, picryl serum was employed for this purpose with avoidance of much of the local toxicity.

RESULTS

1. Induction of Delayed Cutaneous Hypersensitivity to Picryl Chloride by Concomitant Use of the Wax of the Tubercle Bacillus.—Groups of guinea pigs have been treated with picryl chloride under various circumstances in order to establish a comparative basis for assessing the rôle of the wax of the tubercle bacillus in modifying the allergic response.

In order to reproduce the observation that inunction or injection of the chemical into the skin may eventuate in delayed contact hypersensitivity, the following groups were run:

Group 1.—Picryl chloride in olive oil applied to the skin.

Group 2.—Picryl chloride in saline injected intradermally.

In addition, in order to confirm the observation that administration of the substance by non-dermal routes will not result in contact hypersensitivity, two other groups of guinea pigs were employed:

Group 3.—Picryl chloride in saline subcutaneously.

Group 4.—Picryl chloride in saline intraperitoneally.

Further, it was desirable to determine whether an immunologic adjuvant might be capable of causing the drug to induce delayed hypersensitivity following non-dermal administration:

Group 5.—Picryl chloride in water-in-oil emulsion intraperitoneally.

The last group served to test the ability of the purified wax of the human tubercle bacillus to modify the response to picryl chloride in the direction of delayed contact hypersensitivity:

Group 6.—Picryl chloride plus tubercle bacillary wax intraperitoneally.

In the latter four groups of animals where a non-dermal injection route was employed, precautions to avoid skin contamination by the chemical were followed, as described earlier.

The results of subsequent contact and intradermal tests in these six groups of animals were entirely unequivocal. Representative data are shown in Table I. Among the first five groups indications of very moderate hypersensitivity to contact and intradermal application of picryl chloride were seen only in groups 1 and 2, those which had been treated by the dermal route and for prolonged periods of time. Only three of fourteen animals responded; the

TABLE I

Delayed Contact and Intracutaneous Responses to Picryl Chloride in Guinea Pigs Sensitized by Various Methods

Group	Sensitizing treatment	No. of animals	No. of sensitizing treatments	Time after last treatment	No. of animals reactive	Average results of skin tests			
						Contact*		Intracutaneous†	
						24 hrs.	48 hrs.	24 hrs.	48 hrs.
1	PCI applied to skin	8	31-38	10 days	2	0.15 +	0.04 +	11.8 1.5	9.3 1.2
2	PCI intracutaneously	6	26	10	1	0.10 ±	0	13.8 1.6	10.8 1.4
3	PCI subcutaneously	5	4	13	0	0	0	10.6 1.3	8.2 1.0
4	PCI intraperitoneally	5	4	17	0	0	0	11.5 1.5	9.5 1.0
5	PCI + water-in-oil intraperitoneally	8	2-4	17	0	0	0	10.6 1.0	8.6 1.0
6	PCI + T.B. wax intraperitoneally	15	1	13	13	2.5 4+	2.0 4+	21.7 2.1	18.0 1.8
	Controls	12	0	0	0	0	0	9.7 1.4	8.0 1.2

All groups except 6 received additional picryl chloride through periodic skin testing by application and intracutaneous injection. Thus, even groups 3 and 4 received some dermal stimulus (four contact and three intracutaneous tests) before the results recorded above were observed. This amount of skin application did not suffice to induce cutaneous reactivity.

* Contact test readings: The first figure indicates the thickness in millimeters of the indurated skin above the level of surrounding normal skin. The second symbol indicates degree of erythema, 4+ being maximum.

† Intracutaneous test readings: The first figure is average diameter, the second estimated height of the reaction, both in millimeters.

reactions were weak and irregular, but discernible.¹ In groups 3, 4, and 5, in which subcutaneous and intraperitoneal administration had been employed,

¹ These results have been much more moderate than those described in similarly treated guinea pigs by Landsteiner and Chase, and in communications from Dr. Chase. We can only infer a difference in guinea pig stocks. For our present purpose the very modest results of sensitization by the cutaneous route have by contrast emphasized the marked sensitivity established by the addition of wax with another avenue of injection.

in the last instance with admixture of an oily adjuvant, there was at no time any indication of the development of such responsiveness in a total of eighteen guinea pigs. In sharp contrast to these results it was found that thirteen of fifteen animals of group 6, after only one injection of picryl chloride with tubercle bacillary wax intraperitoneally, developed marked delayed sensitivity to subsequent contact or intradermal tests with the chemical. The contact sites were intensely erythematous and in most instances the skin was elevated by induration to 3 mm. Intracutaneous test sites showed the slow development and general characteristics of tuberculin reactions, and in six of the instances listed in the table these were accompanied by central necrosis. Two of these animals failed to react (these negative results are included in the averages given in the table), and one of these likewise failed after a second injection.

The figures listed in the table fail to convey adequately the differences among the groups described. Wax-treated animals 24 hours after testing could be recognized at a glance. These reactions are illustrated in color in Figs. 1 and 2.

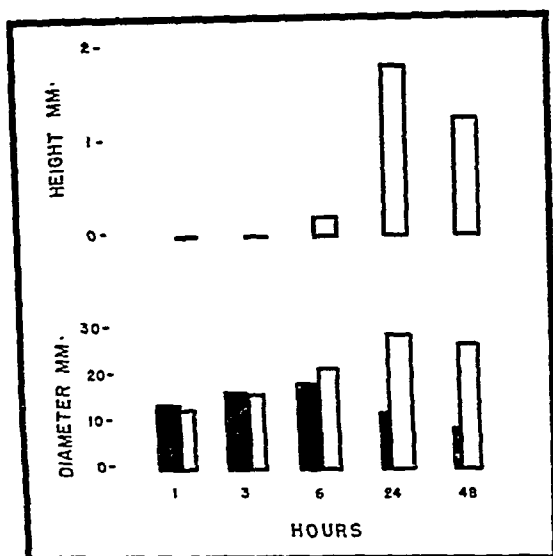
It was found that the level of reactivity of the wax-treated animals was not intensified by more injections, to a total of three. The hypersensitive state is persistent once established, for five reactive animals tested at 164 days after a single sensitizing injection showed contact and intracutaneous reactions of the same degree as those seen at 13 days after the sensitizing treatment.

2. *Characterization of the Delayed Hypersensitivity Induced by Picryl Chloride Plus Wax.*—The delayed contact reactivity established by the use of tubercle bacillary wax with picryl chloride is of high degree and appears to depend upon a specific qualitative effect of the wax rather than a simple adjuvant activity, as indicated by the failure of water-in-oil emulsion to cause the same responsiveness to the antigen.

It was of interest now to characterize the delayed hypersensitivity *per se* and also in relation to other immunologic responses of the animal, particularly antibody production and anaphylactic reactivity. The delayed hypersensitive reaction should bear no relationship either to humoral antibody occurrence or level, or to immediate hypersensitivity of an anaphylactic or Arthus nature.

(a) *Chronological Development of the Delayed Cutaneous Reaction.*—It should be said at the outset that we have never observed a cutaneous reaction of the Arthus type in any of the animals listed in Table I, even though repeated tests were made at intervals after succeeding injections or applications. These animals either showed delayed responses to contact and intracutaneous injection of the picryl chloride, or no response at all to either test. When the reactions were positive, they showed the chronological development characteristic of the tuberculin type of reaction. This is portrayed in Text-fig. 1, in which the upper row of blocks represents the average development of contact responses in five guinea pigs sensitized by one intraperitoneal injection of picryl

chloride with wax 90 days earlier. At 6 hours, two of the five animals showed a faint beginning erythema and induration. Full development of the reaction was seen in all guinea pigs at 24 hours. The lower blocks depict simultaneous intracutaneous tests carried out on the opposite flanks of the same animals. Since picryl chloride is moderately irritating to normal tissues, the average reactions of three control animals similarly tested are shown also. In the wax-treated animals the intracutaneous responses parallel the contact tests



TEXT-FIG. 1. Timed readings of contact and intracutaneous tests with picryl chloride.

Upper. Contact tests in guinea pigs sensitized with picryl chloride plus tubercle bacillary wax. Height of block indicates thickness of induration in millimeters.

Lower. Intracutaneous tests in the same group of animals. Height of block represents diameter of reaction in millimeters, the width represents thickness of induration.

Black boxes indicate results seen in normal animals due to the irritative activity of picryl chloride.

in their development; at 6 hours there is hardly a significant indication of incipient reaction, while at 24 hours this is marked, at a time when the traumatic response in control animals has subsided. In both contact and intracutaneous responses there is seen to be some diminution at 48 hours. This was a rather regular but not invariable occurrence, and has been noted by us also in tuberculin tests in guinea pigs.

The absence of significant reactivity in the animals of the groups other than those treated with wax could be related to a failure of the picryl chloride to act as an effective antigen. That this was not the case is illustrated in Table II and discussed in the following section.

TABLE II

Correlation of Serological Responses with Delayed Cutaneous Reactivity to Picryl Chloride

Group	Sensitizing treatment	Guinea pig No.	No. of treatments	Time since last treatment	Complement fixation titer	24 hr. contact test
				days		
1	PCl applied to skin	C-60	31	10	0	0
		C-64	31	10	16	0
		C-65	31	10	16	0
		D-51	24	9	8	0
		D-55	24	10	0	0.3 ±
		D-58	24	9	1	0
2	PCl intracutaneously	E-86	20	15	16	0
		E-87	20	15	8	0
		E-91	20	15	16	0
3	PCl subcutaneously	C-66	3	14	4	0
		C-68	3	14	8	0
		C-70	3	14	0	0
		C-71	3	14	32	0
		C-73	3	14	16	0
4	PCl intraperitoneally	C-26	2	46	16	0
		C-27	3	14	4	0
		C-28	3	14	16	0
5	PCl + water-in-oil intra-peritoneally	C-88	3	13	4	0
		C-58	3	13	16	0
		C-89	3	13	2	0
		C-90	3	13	16	0
		D-49	2	13	1	0
		D-53	2	13	0	0
		D-56	2	13	0	0
6	PCl + T.B. wax intraperitoneally	C-29	2	39	8	3.0 +
		C-33	3	14	64	1.5 +
		D-48	2	14	16	1.0 +
		D-68	1	26	4	2.5 +
		F- 5	1	14	1	2.5 +
		F- 6	1	14	1	1.0 ±
		F- 7	1	13	8	4.0 +
		F- 8	1	13	32	1.0 ±
		F- 9	1	13	8	0
		F-10	1	13	32	4.0 +
	Controls	N- 1	0		0	0
		N- 2	0		0	0
		N- 3	0		0	0
		N- 4	0		0	0
		N- 5	0		0	0
		N- 6	0		0	0

(b) *Lack of Relationship of Delayed Cutaneous Reactivity to Humoral Antibody.*—It is recognized that Arthus reactivity in its occurrence and intensity is directly related to the level of circulating antibody (27-31), while delayed hypersensitive responses are entirely independent of humoral antibody. It was therefore desirable to determine whether the cutaneous reactions described here could be dissociated from humoral antibody in the picryl chloride-wax-treated animals. In addition, it was necessary to know that animals in those groups without skin reactivity had, nevertheless, received an immunologic stimulus from the picryl chloride applied or injected. Sera from animals of all groups were titrated against picrylated serum by the complement fixation method. On the day previous to bleeding all animals received contact skin tests. The correlated results of these antibody and skin tests are shown in Table II. Most of the animals in all groups responded to picryl chloride with the elaboration of antibody, but at the time of these tests only the animals of group 6, sensitized with picryl chloride plus wax, showed significant contact reactivity of the skin. Yet this group of guinea pigs did not produce significantly greater amounts of measurable antibody than did those which failed to develop skin reactivity. Furthermore, within this group there is no correlation of intensity of skin response with titer in any individual instance. It may be concluded, therefore, that the cutaneous reactivity induced by picryl chloride plus wax is independent of humoral antibody, that the failure of picryl chloride, in other combinations and by other routes, to induce such sensitivity was not due to a lack of antigenic activity of this substance, and finally that the wax, whatever its effect may be, does not act as an immunologic adjuvant in intensifying immunologic responses as judged by antibody titers. The last conclusion was arrived at from experiments with tuberculo-protein antigen (2) and with egg albumin (32) also.

(c) *Lack of Relationship of Delayed Cutaneous Reactivity to Anaphylactic Reactivity.*—In order further to demonstrate that the reactivity observed in the picryl chloride-wax-treated animals is delayed in character, an experiment was carried out to dissociate this reactivity from anaphylaxis. Landsteiner and Chase (12) have shown that guinea pigs with simultaneous reactivities can be desensitized more readily to the anaphylactic type, so that the delayed cutaneous form remains intact.

A desensitization experiment was carried out as follows:—

Guinea pigs of all groups were skin tested by the contact and intradermal methods. Twenty-four hours later, part of each group received 0.25 ml. (containing 4.6 mg. dry residue weight) of picrylated serum subcutaneously in the dorsal nuchal area, a second injection 4 hours later into the dorsal lumbar area, a third 3 hours later into the sacral area, and a fourth the next morning in the dorsal thoracic region. The four injections, totaling 18.4 mg. of material, were completed in a period of 24 hours. Several hours after completion of the desensitizing injections all animals were again skin tested by the contact and intracutaneous methods. On the following day the 24 hour skin reactions were read and intravenous tests (saphenous

vein) for anaphylaxis were carried out by the injection of 1 ml. (18.5 mg.) of picrylated serum. The results are shown in Table III.

The experiment was carried out sufficiently long after the last sensitizing injections so that spontaneous loss of anaphylactic sensitivity had probably occurred in some of the animals. Previous observers (12) have indicated that anaphylactic sensitization to picryl chloride may diminish considerably by the 5th week after a sensitizing injection. Whether desensitization was spontaneous or induced by the procedure employed is of no consequence to the essential points which emerge from this experiment, however. Spontaneously or experimentally desensitized animals exhibited well developed cutaneous reactivity in the absence of any indication of anaphylactic responsiveness. Conversely undesensitized guinea pigs without cutaneous reactivity showed anaphylactic shock. The independence of the two types of hypersensitive response to the same antigen is thus evident.

(d) *Failure of Serum to Transfer the Delayed Cutaneous Reactivity Passively.*—The well known inability of serum to convey delayed hypersensitivity to normal recipient animals is one of the basic features distinguishing this from the hypersensitivities of the immediate type. Chase (14) has recently indicated the most favorable circumstances for the transfer of the immediate form of reactivity. In the present experiment the various factors were so arranged as to provide such circumstances.

Well nourished albino guinea pigs weighing between 350 and 400 gm. were employed as recipients. Into the skin of one flank of each animal 0.2 ml. of a test serum was injected. The same quantity of another test serum was injected in the opposite flank. Each serum was given to two guinea pigs. Thus, each animal received two different sera, and each serum was tested in two different animals. Forty-eight hours later all animals received 2 mg. of picryl chloride in 0.5 ml. of olive oil subcutaneously in the ventral abdominal region. Readings of the serum injection sites were made at 15 minutes and at 1, 2, 3, 6, and 24 hours after injection of antigen. The summarized results of these tests are shown in Table IV along with the cutaneous reactivities of the donor animals at the time of serum collection and the titers of these sera in complement fixation tests.

As has been mentioned earlier, no clear cut evidence of Arthus reactivity has been obtained in any of our groups of animals, and it is therefore not surprising that such reactivity was only an occasional occurrence in the transfer test guinea pigs. The figures set forth in the table indicate the infrequency of such reactions, and furthermore show that:

(a) All passive reactions occurred early; none persisted to 24 hours, a time when the delayed cutaneous reaction reaches its apex.

(b) The transfer of the immediate form of reactivity bears no relationship to the occurrence of delayed cutaneous reactivity in the donor animals, either with regard to groups or in individual cases.

(c) There was no relationship between the complement fixation titer of a

TABLE III

Dissociation of Delayed Cutaneous from Anaphylactic Hypersensitivity by Desensitization

Group	Guinea pig No.	No. of injections or applications	Time since last treatment	Contact test* before desensitization	Contact test* after desensitization	Anaphylactic reaction
Desensitized						
6. PCI plus wax intraperitoneally	F- 7	1	162	1.0 +	2.0 +	0
“ “ “ “	F- 8	1	162	0.5 +	0.3 +	0
“ “ “ “	F-10	1	162	3.0 +	2.0 +	0
2. PCI intracutaneously	E-87	26	82	0.5 +	0.3 ±	0
“ “	E-91	26	82	0	0	0
5. PCI in water-oil intraperitoneally	C-58	4	89	0	0	0
PCI in water-oil intraperitoneally	C-88	4	89	0	0	0
PCI in water-oil intraperitoneally	C-90	4	89	0	0	0
Not desensitized						
6. PCI plus wax intraperitoneally	F- 5	1	162	2.0 +	2.0 +	4+
2. PCI intracutaneously	E-90	26	82	0.3 +	0.5 ±	4+
1. PCI on skin	E-62	25	82	0 ±	0 ±	0
“ “ “	E-63	25	82	0 ±	0 ±	0
“ “ “	D-58	31	82	0	0	0
“ “ “	E-61	25	82	0	0	0
4. PCI intraperitoneally	C-28	4	89	0	0	3+
5. PCI in water-oil intraperitoneally	D-49	4	89	0	0	4+
PCI in water-oil intraperitoneally	D-53	3	89	0	0	0
2. PCI intracutaneously	E-93	26	82	0	0	2+
3. PCI subcutaneously	C-73	4	89	0	0	0
6. PCI plus wax intraperitoneally	F- 6	1	162	0	0	0
“ “ “ “	F- 9	1	162	0	0	0
Normal controls						
	N-11§	—	—	0	0	0
	N-12§	—	—	0	0	0
	N-13§	—	—	0	0	0
	C-14	—	—	0	0	0
	B-85	—	—	0	0	0

* 24 hour readings.

† 4+ indicates acute anaphylactic death.

§ These normal animals were subjected to the desensitization procedure.

serum and the transfer of immediate hypersensitivity in any individual case.

The central point is the failure of animals with well developed delayed cutaneous reactivity to picryl chloride to transfer this passively *via* serum.

3. *Conditions Governing the Activity of Tubercle Bacillary Wax in Its "Directive" Role in Hypersensitivity.*—It would be of much interest to define the circumstances under which wax may act in the capacity described, and the mechanism of its activity. Dienes in his earlier observations on the injection of

TABLE IV
Passive Transfer Tests

Group	No. of guinea pigs in group	No. of infections or applications	Time since last treatment	Positive skin reactions at time of bleeding	Positive complement-fixing antibodies	Positive transfer sites at*			
						1 hr.	3 hrs.	6 hrs.	24 hrs.
1			days						
PCI applied to skin	15	18-31	10-15	0/15	13/15	5/30	3/30	2/30	0/30
2									
PCI intracutaneously	3	20	15	0/3	3/3	0/6	1/6	0/6	0/6
3									
PCI subcutaneously	3	3	14	0/3	2/3	0/6	0/6	0/6	0/6
4									
PCI intraperitoneally	3	3	14	0/3	3/3	0/6	0/6	0/6	0/6
5									
PCI in water-oil intraperitoneally	7	3	13	0/7	5/7	2/14	2/14	1/14	0/14
6									
PCI plus wax intraperitoneally	9	1-3	14	8/9	9/9	1/18	1/18	2/18	0/18
Normal animals	3	—	—	0/3	0/3	0/6	0/6	0/6	0/6

* Numerator indicates positive reactions, denominator total tests (two tests for each serum).

various antigenic substances into tuberculous guinea pigs (18) found that administration of the antigens directly into foci of infection was most favorable if not essential for the development of altered hypersensitive reactions. It seems dubious that the tuberculous cellular response in such areas was the essential determinant, since Hanks (20) found that if infection were initiated in the testicle, egg albumin injected into the area within 18 hours, and the testicle removed 6 to 12 hours later, altered reactivity to the antigen eventuated.

In the work reported here, picryl chloride and wax were injected into the peritoneal cavity in succession, so that these were in intimate contact within the limits permitted by a cavity of this surface area, and by the volumes of fluid injected (wax in 0.5 ml., picryl chloride in 1.0 ml.). We have thus far

made only tentative attempts to delimit the conditions under which the rôle of wax can be exercised, and so far as these go they indicate that this substance and antigen must be in rather close proximity in the tissues in order for the effect to become apparent. Preliminary experiments in this connection are the following:

(a) Six guinea pigs were treated by four daily applications of picryl chloride in olive oil to the skin. The day following the last application, 5 mg. of wax was given intraperitoneally to each animal, and on subsequent days fourteen more applications of picryl chloride were made to the skin. After a pause of 10 weeks, a series of seven more applications was made. The results of this treatment were negative. This contrasts sharply with the result of the same single dose of wax along with picryl chloride, both into the peritoneal cavity.

(b) Six guinea pigs were treated by daily application of picryl chloride and wax in olive oil to the skin. The picryl chloride was present in a concentration of 15 mg. per ml., the wax 10 mg. per ml. The one drop employed for each application contained approximately 0.75 mg. of picryl chloride and 0.5 mg. of wax. After twenty-four daily inunctions, these animals showed in two instances very mild contact reactions, a result similar to that following treatment of the skin with picryl chloride alone. Since application of wax to the skin produced no visible response in this tissue, it might be inferred that a cellular response to this material is of some importance in determining the kind of hypersensitive reactivity set up in the body. On the other hand, the failure may be due to impermeability of guinea pig skin to the wax molecules.

(c) Six guinea pigs which had been infected with virulent human bacilli by the subcutaneous and intracutaneous routes in the left inguinal area 4 weeks earlier, and which showed only beginning dissemination of the disease as judged by autopsies of similarly infected animals, were treated by eight daily applications of picryl chloride to the skin. The last application resulted in the development of good contact responses in all animals. On retest 2 weeks later the responses were much diminished, but again unanimous. This suggests that the distribution of bacilli, and consequently of wax, more widely through the body as the result of generalized infection may serve as a conditioner for the development of altered reactivity to antigen no matter where it gains access to the tissues.

This experiment, as well as the early experiences of Dienes and others (16-21) with injections of antigens into tuberculous animals, leads to a consideration of "heteroallergic" phenomena in tuberculosis. Weissfeiler (33), Higginbotham (34), and others have observed the reactions of tuberculous guinea pigs to cutaneous injections of *E. coli*, *Staphylococcus aureus*, *Brucella suis*, diphtheroids, *Actinomyces*, and *Sarcinas*. Such responses appear to have the characteristics of the Koch phenomenon. Rich (35) has described a patient dying of staphylococcic septicemia; at autopsy, about the periphery of a fibro-

caseous tuberculous pulmonary lesion, a hemorrhagic reaction to the staphylococcus was found. It seems a possibility that such phenomena may be explicable not as "heteroallergic" responses on an immunologically non-specific basis, but as the result of the marked tendency of the wax portion of the tubercle bacillus to cause tissues to respond with tuberculin-type reactions to various antigenic substances. In the present paper this is exemplified by the use of picryl chloride.

DISCUSSION

Picryl chloride is a substance which, although antigenic to the animal body by any parenteral route, induces delayed hypersensitivity only when it gains entrance through the skin either by application or intracutaneous injection. Under these specialized conditions a moderate level of delayed contact reactivity develops in a proportion of guinea pigs treated.

Because in previous work (1, 2) we had observed that the wax fraction of the tubercle bacillus possesses the property of causing the animal body to respond to tuberculo-protein with typical tuberculin allergy, it seemed reasonable to propose that this lipid might have similar "directive" properties in altering the type of hypersensitivity to other antigenic substances. It is demonstrated in the present report that such an effect is markedly evident when picryl chloride is employed as antigen, injections of this and the wax being given by a route (intraperitoneal) which does not result in delayed hypersensitivity when the picryl chloride alone is employed. Responses in picryl chloride-wax-treated animals are much more regular and far more intense than those which follow sensitization with picryl chloride *via* the only "natural" route available, the skin. Previous observations of a similar effect of killed tubercle bacilli (15) in inducing this same altered hypersensitivity are thus referable to an isolated lipoidal constituent of the organism which, if injected with antigenic substances, possesses the biological property of causing delayed allergic responses to the antigens themselves.

The delayed nature of the reactivity induced in this manner is evidenced by the chronological and morphological character of the cutaneous response, its lack of relationship to humoral antibody level, its failure to be passively transferred to normal recipients by serum, and its independence of the anaphylactic state.

The specific activity of the wax is indicated, as in previous experiments on tuberculosis (2) and with egg albumin (32), by the failure of an ordinary immunologic adjuvant, represented by water-in-oil emulsion, to effect a similar change in hypersensitive responsiveness. Nor could other workers (13, 15) effect such a change by intraperitoneal injections of other adjuvants including alumina, tapioca, and charcoal, along with picryl chloride. Furthermore, the wax has given no evidence of being an immunologic adjuvant, since with three

antigens in our own experience it has in no instance caused increased antibody production over that seen in animals receiving the antigens alone.

The mechanism of activity of this bacterial wax is not known. The marked histologic response occasioned by the substance would seem to be unrelated to this activity, for similar cellular responses are occasioned by another lipid—the phosphatide—of the same organism, and this does not influence the hypersensitive response. Furthermore, as Rich (36) has pointed out, the bacterial type of delayed allergy occurs in diseases in which no lesion comparable to that of tuberculosis occurs at all.

So far as available evidence is concerned, it seems necessary that antigen and the lipid must be in rather intimate contact within the body in order for the alteration in hypersensitive response to occur. Observations derived from work with another antigen (32) indicate that several hours may intervene between injection of wax and antigen into the same site. It is of interest, however, that the tuberculous animal, in which the bacillus and its lipids are disseminated through the body, may provide conditions for the establishment of the marked delayed type of hypersensitivity as the result of applications of picryl chloride to the skin. It is pertinent to speculate whether in the human being with quantitatively sufficient infection there may be a tendency to development of delayed hypersensitivity to any antigenic substance which, in the uninfected individual, would cause a form of immediate allergic reactivity. Involved in this consideration also is the question of so called “heteroallergic” reactivity described in tuberculous animals (33, 34) and the human being (35), wherein bacteria unrelated to the tubercle bacillus may provoke reactions analogous to the Koch phenomenon. It is entirely conceivable that under the influence of the tubercle bacillary wax responses to the antigens of these bacteria would develop as forms of delayed hypersensitivity.

It may be questioned how the demonstrated activity of tubercle bacillary wax bears any relationship to the spontaneous occurrence of delayed contact reactivity to picryl chloride or other simple chemical substances. In the absence of the wax described, these must act through the skin in order to sensitize effectively. This special circumstance may have an analogy in the case of tuberculin reactivity, for here it is necessary that the entire bacillus be present in the tissues for sensitization to eventuate. A common factor may be involved in both of these cases, for we find that the same component of the tubercle bacillus which causes delayed hypersensitivity to isolated tuberculoprotein to become established also permits delayed hypersensitivity to picryl chloride without the intermediation of the skin. Perhaps then a lipid with activities similar to those of tubercle bacillary wax is present as a cellular component of skin. When released by injury (most substances inducing contact dermal hypersensitivity are primarily irritating) such lipids may function in the same manner as does the bacillary wax. This possibility is being investigated.

SUMMARY

The purified wax fraction of the tubercle bacillus, which has been previously demonstrated as an essential element in causing delayed tuberculin hypersensitivity in response to the protein of the tubercle bacillus, is now found to have the same activity with regard to a simple chemical antigen, picryl chloride. One injection of this compound with wax intraperitoneally into guinea pigs results in a marked delayed cutaneous hypersensitivity, demonstrable by contact and intracutaneous test, and of long duration. The effect is not related to an adjuvant activity of the wax as defined by ordinary standards.

The relationship of these observations to the occurrence of "heteroallergic" phenomena in tuberculosis is discussed.

The possibility that the occurrence of spontaneous contact hypersensitivities may depend upon the presence of similarly active lipoidal components of the skin is commented upon.

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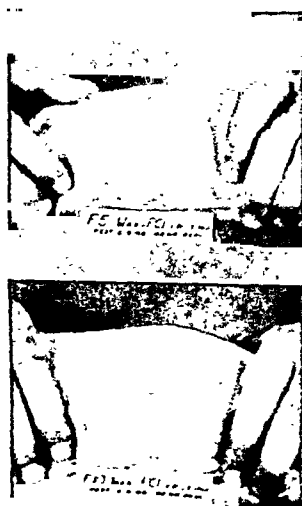
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EXPLANATION OF PLATE 23

FIG. 1. Contact test 90 days following one injection of picryl chloride plus wax intraperitoneally. 48 hour reaction.

FIG. 2. Intracutaneous test under the same conditions as above. 48 hour reaction.



(Raffel and Forney: Tubercle bacillus wax and delayed hypersensitivity)

A HEMOLYSIN ASSOCIATED WITH THE MUMPS VIRUS* ‡

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During the course of unpublished experiments by Levens and Enders, hemolysis was observed in suspensions of chicken erythrocytes exposed at 37°C. to amniotic or allantoic fluids derived from chick embryos infected with mumps virus.¹ The results of systematic studies of this hemolytic factor are described in the present communication.

Methods

Preparation of Virus Suspensions.—A strain of mumps virus which had been carried for 42 passages in eggs was used. Infected amniotic fluid was inoculated into the amniotic sac of 7-day-old embryos which were then incubated at 35°C. for 6 to 8 days. At the end of this time the amniotic and allantoic fluids were removed and pooled. Aliquots were placed in glass ampoules, sealed, and stored in the dry ice cabinet until used. Allantoic fluids withdrawn from embryos 48 hours after inoculation by the allantoic route with the PR8 and Lee B strains of influenza virus were also employed in certain experiments.

Titration of Hemolysin.—Serial twofold dilutions of infected amniotic fluids were prepared in isotonic phosphate buffer (pH 7.0–7.2). To 0.5 cc. of each dilution was added an equal volume of a suspension in the buffer of chicken erythrocytes in concentrations of 1 per cent, 2 per cent, or 4 per cent. Only 2 per cent or 4 per cent suspensions of cells were used in most experiments, since they were found to give more uniform results. In the determination of hemagglutination, the procedure of Hirst (1) was employed except in one instance when that of Salk (2) was used. After 1 hour at 4°C. the degree of hemagglutination was noted and the tubes were gently agitated to resuspend the agglutinated red cells. They were then incubated for 2 to 4 hours at 37°C. Following incubation, the tubes were again very cautiously agitated and were centrifuged at 1500 R.P.M. The supernatant fluids were poured off, diluted with 9 volumes of distilled water, and their content of hemoglobin determined. The degree of

* Certain of the data described in this paper were presented at a Symposium on Mumps held on May 12, 1948, in Minneapolis, at a meeting of the Society of American Bacteriologists.

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¹ The first observations on this hemolytic factor were made with a strain of mumps virus which had been subjected to 35 egg passages. Since then hemolysin has been demonstrated in the amniotic fluid of the 5th egg passage of this same strain and in three other strains isolated directly from the saliva of mumps patients by embryonic inoculation and carried for 4 or 5 passages in eggs.

hemolysis was calculated with reference to a control standard. This standard was prepared with the same volume of cells completely hemolyzed by suspension in distilled water and addition of a drop of dilute ammonium hydroxide. The hemoglobin determinations were made with an Evelyn photoelectric colorimeter using filter 540 (3).

EXPERIMENTAL

Comparison of the Hemolytic and Hemagglutinative Activity of Mumps and Influenza Viruses

Allantoic fluids infected respectively with mumps, PR8, and Lee B influenza viruses were titrated, using a 1 per cent suspension of chicken erythrocytes. Hemagglutination was recorded after 1 hour at 4°C. and hemolysis after 4 hours at 37°C. The results of these titrations are presented in Table I. They show

TABLE 1

Hemagglutinative and Hemolytic Activity of Mumps and Influenza Viruses on Chicken Erythrocytes

Infected fluid	Test	Dilutions of fluid									Buffer control
		8	16	32	64	128	256	512	1024	2048	
Mumps	Hemagglutination	3*	3	2	1	1—	0	0	0	0	0
	Hemolysis	48†	43	29	25	13	0	0	0	0	0
PR8	Hemagglutination	4	4	3	3	2	2	1	1	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0	0
Lee B	Hemagglutination	4	3	2	2	2	1	1	1—	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0	0

* 1 per cent chicken cells. Hirst technique. Figures denote degree of hemagglutination.

† Per cent of cells hemolyzed determined colorimetrically.

that fluids infected with mumps virus agglutinate chicken red cells at refrigerator temperatures and, on subsequent incubation at 37°C., hemolyze them to about the same extent. In contrast the influenza viruses readily agglutinate these erythrocytes but produce no hemolysis. In these and other experiments, the titers of hemagglutinin and of hemolysin in preparations of mumps virus were usually of the same order. Occasionally, however, a fluid might exhibit considerable hemagglutinative activity but little hemolytic effect.

Comparative Activity of the Hemolysin on Chicken, Sheep, and Human Erythrocytes

Four per cent suspensions of freshly obtained chicken, sheep, and human type O erythrocytes were prepared in phosphate buffer. Allantoic fluid infected with mumps virus was diluted and its hemolytic titer simultaneously deter-

mined against each type of cell suspension. At the same time the hemagglutinative titer was determined with an 0.25 per cent suspension of the cells of each species. The method of Salk was employed instead of that of Hirst because tests with human erythrocytes are unsatisfactory by the latter method. The results are shown in Table II.

It is apparent that although the erythrocytes of these three species are all susceptible to hemolysis, those of man are less affected than the cells of the sheep or the chicken. The human cells also proved to be less sensitive to hemagglutination by the virus.

TABLE II

Hemolytic Effect of Mumps-Infected Allantoic Fluid on Erythrocytes of Various Species

Species	Test	Cell concentration	Dilutions of fluid								Buffer control
			8	16	32	64	128	256	512	1024	
		per cent									
Chicken	Hemagglutination	0.25	+	+	+	+	+	±	±	0	0
	Hemolysis	4.	44‡	36	26	12	7	5	1	0	0
Sheep	Hemagglutination	0.25	+	+	+	+	+	±	±	0	0
	Hemolysis	4.	18	18	13	8	5	3	0	0	0
Human	Hemagglutination	0.25	+	+	+	+	±	0	0	0	0
	Hemolysis	4.	22	17	6	1	0	0	0	0	0

* Salk technique. + denotes complete agglutination of cells.

‡ Per cent of cells hemolyzed as determined colorimetrically.

Effect of Physical and Chemical Agents on the Hemolytic Activity of Mumps Virus

Inactivation by Heat.—Fluids infected with mumps virus were placed in sealed glass ampoules and completely immersed in a water bath at various temperatures for varying periods of time. Afterwards they were immediately placed in ice water. The concentrations of hemagglutinin and hemolysin in each specimen were then determined. These, together with that of the starting material, are recorded in Table III.

The data show that the hemagglutinative and hemolytic activity of the virus are destroyed after 30 minutes at 55°C.; both properties are markedly impaired after 30 minutes at 50°C.; but exposure for 10 minutes at this temperature almost completely destroys the hemolytic activity while leaving the hemagglutinin almost intact.

The hemolysin also seems to be less stable at low temperatures. A pool of

infected amniotic fluid stored at -20°C . was thawed and refrozen four times during an interval of 3 weeks. At the end of 3 days after the beginning of the period of storage the concentration of hemolysin had decreased and by the end of 3 weeks no hemolysin could be detected, whereas the hemagglutinin titer remained unchanged.

Effect of Temperature and Time of Incubation on Hemolysis.—To determine the effect of temperature and time of incubation on the degree of hemolysis,

TABLE III
Inactivation of the Hemolytic Activity of Mumps Virus by Heat

Mumps-infected fluid	Temperature	Time	Test	Dilutions of serum								Buffer control	
				4	8	16	32	64	128	256	512		1024
Allantoic	°C.	min.											
	Unheated	—	Hemagglutination Hemolysis	3* 29‡	3 36	3 29	3 29	2 20	2 10	± 4	0 1	0 0	0 0
	50	30	Hemagglutination Hemolysis	1— 5	1— 0	1— 0	1— 0	± 0	0 0	0 0	0 0	0 0	
	55	30	Hemagglutination Hemolysis	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	
	Unheated	—	Hemagglutination Hemolysis	4 53	4 51	4 46	3 40	2 29	1 20	1 15	± 2	0 0	0 0
	Amniotic	50	10	Hemagglutination Hemolysis	4 6	4 5	3 5	2 ±	1 0	1 0	± 0	0 0	0 0
50		20	Hemagglutination Hemolysis	4 5	4 0	3 0	2 0	1 0	1 0	± 0	0 0	0 0	0 0

* 2 per cent chicken cells. Hirst technique. Figures denote degree of hemagglutination.

† Per cent of cells hemolyzed as determined colorimetrically.

amniotic fluids infected with mumps virus were mixed with 4 per cent suspensions of chicken red blood cells and incubated at various temperatures for varying periods. Unless otherwise noted, the suspensions were first placed at 4°C . for 1 hour, shaken, and then incubated at 37°C . Table IV provides a summary of the results obtained in these experiments. They indicate that most of the hemolysis occurs during the 1st hour of incubation at 37°C . This temperature appears to be optimal, since less hemolysis was observed at 25°C . and none at 4°C . during the same interval. These data also show that preliminary chilling for 1 hour at 4°C ., which permits marked hemagglutination, is not essential for the subsequent occurrence of hemolysis at 37°C . In fact, it has been found that

when virus and red cells are mixed and maintained at 37°C., conditions which are attended by little observable hemagglutination, maximal hemolysis ensues. These observations taken together suggest that hemagglutination is not an essential factor in the hemolytic process.

Effect of pH on Hemolysis.—The effect of different hydrogen ion concentrations on the activity of the hemolysin was determined by titrations carried out in isotonic phosphate buffers of varying pH values. To allow for the fact that

TABLE IV

Effect of Time and Temperature of Incubation on the Degree of Hemolysis Produced by Mumps Virus

Mumps-infected fluid	Reaction	Temperature	Time	Dilutions of fluid										Buffer control
				4	8	16	32	64	128	256	512	1024		
Amniotic I	Hemolysis	37	hrs.											
			1	19*	16	14	9	7	nd	0	0	0	0	
			2	17	19	14	8	7	1.2	0	0	0	0	
			3	22	19	15	8	4	2	1	0	0	0	
Amniotic II	Hemolysis	4	4	0	0	0	0	0	0	0	0	0	0	
			25	4	12	10	8	0	0	0	0	0	0	
			37	4	33	27	19	10	6	4	0	0	0	
Amniotic III	Hemagglutination	{	4†	1	4	4	2	2	1—	1—	0	0	0	0
	Hemolysis		37	2	48	42	30	26	16	8	0	0	0	0
	Hemagglutination		37§	1	0	0	0	0	0	0	0	0	0	
	Hemolysis		37	2	48	40	35	22	16	5	0	0	0	0

nd, not done.

* Per cent of cells hemolyzed determined colorimetrically. 4 per cent suspension.

† 2 per cent cells used for experiments with amniotic fluid III. Hirst's technique. Figures denote degree of hemagglutination.

§ No initial chilling at 4°C. Virus dilutions and red blood cells mixed at 37°C.

red blood cells themselves exert a considerable buffering action, the pH values at which the reaction occurred were determined after incubation for 4 hours at 37°C. The results, presented graphically in Fig. 1, indicate that the hemolytic activity appears to be maximal between pH values of 7.0 and 8.0. Below pH 6.5 or above pH 8.5 little or no activity was observed. In contrast the hemagglutinin was not significantly affected over the range of hydrogen ion concentrations which was employed.

Effect of Composition of Buffer on Hemolysis.—Dilutions of an infected amniotic fluid were prepared in monobasic sodium and phosphate buffer (pH 7.2) containing sodium chloride and in a sodium citrate buffer (pH 7.4). To each

were added chicken red cells diluted in the homologous buffer. The titer and degree of hemolysis observed in each buffer system were essentially the same.

Adsorption and Elution of the Hemagglutinin and the Hemolysin by Chicken Red Blood Cells

To determine whether the hemolytic activity could be adsorbed and eluted from chicken red cells like the hemagglutinin (4), 1 cc. of infected amniotic fluid was added to 5 cc. of 10

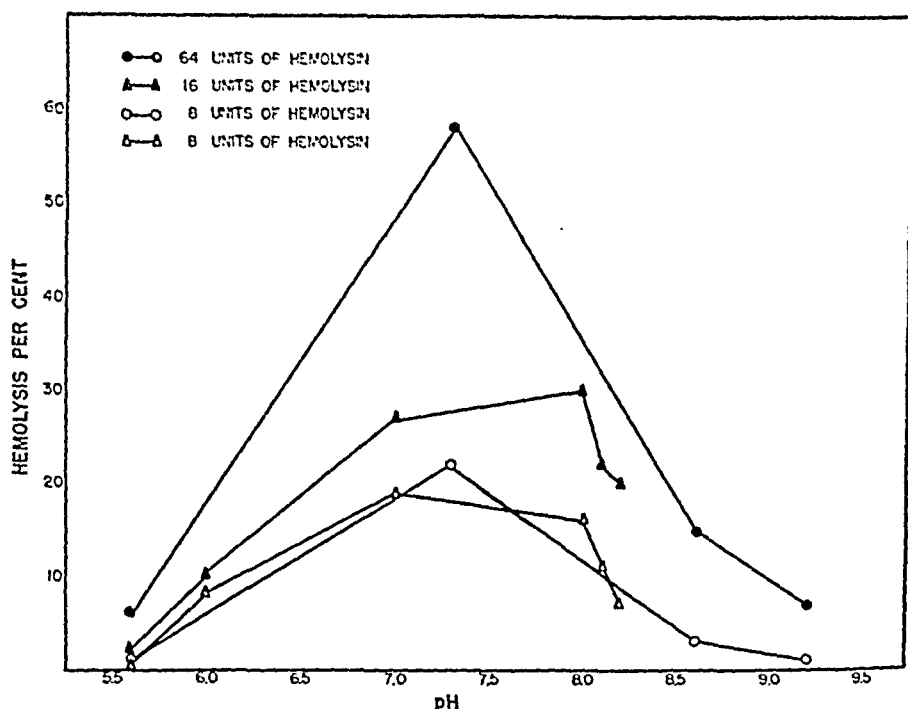


FIG. 1. Effect of pH on the activity of mumps hemolysin. A unit of hemolysin is determined by the ratio: $\frac{\text{Endpoint of hemolytic effect}}{\text{Dilution of fluid used}}$.

per cent chicken cells in saline. The mixture was allowed to stand for 1 hour at 4°C. After removal of the supernatant fluid aliquots of the cells were resuspended in phosphate buffer at pH 6.0 and pH 7.2 respectively and then placed in a water bath for 2 hours at 37°C. These eluates were then separated from the cells by centrifugation and the cells washed twice in buffer (pH 7.2) and resuspended in a concentration of 2 per cent in buffer. The supernatant fluid and eluates were tested for hemolytic activity after dilution in buffer and addition of an equal volume of 2 per cent red cells. To allow for the hemolysis which occurred during elution, the eluate was diluted in buffer, and the concentration of hemoglobin determined in the photoelectric colorimeter. This value was then used to correct the readings obtained after incubation of the eluate with untreated chicken cells. The cells from which the virus was eluted were washed three times in buffer and made up in buffer (pH 7.2) as a 2 per cent suspen-

sion. These cells were then tested for susceptibility to hemolysis by adding to them dilutions of fresh virus suspensions.

TABLE V

The Adsorption and Elution of the Hemagglutinative and Hemolytic Activity of Mumps Virus with Chicken Red Cells

Fluid	Test	Dilutions of fluids*								Buffer control
		12	24	48	96	192	384	768	1536	
Original										
Amniotic fluid	Hemagglutination	4	3	2	1	1	±	0	0	0
	Hemolysis	46†	40	29	20	15	2	0	0	0
Supernatant (pH 6.0) after 1 hr. at 4°C.	Hemagglutination	0	0	0	0					0
	Hemolysis	0	0	0	0					0
Eluate 1 (pH 6.0) after 2 hrs. at 37°C.	Hemagglutination	4	2	±	0	0	0	0	0	0
	Hemolysis	15	11	7	2	0	0	0	0	0
Supernatant (pH 7.2)	Hemagglutination	0	0	0	0					0
	Hemolysis	0	0	0	0					0
Eluate 2 (pH 7.2)	Hemagglutination	4	4	1—	0	0	0	0	0	0
	Hemolysis	12	16	14	4	0	0	0	0	0

* In terms of original fluid before addition of adsorbing chicken red cells.

† Per cent of hemolyzed cells as determined colorimetrically with 2 per cent cells.

Cells	Test	Dilutions of virus								Buffer control
		2	4	8	16	32	64	128	256	
Untreated	Hemagglutination	4*	4	2	2	1	±	0	0	0
	Hemolysis	51†	45	43	35	30	14	6	0	0
Eluted 1	Hemagglutination	±	±	0	0	0	0	0	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0
Eluted 2	Hemagglutination	0	0	0	0	0	0	0	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0

* 2 per cent suspension of cells. Hirst technique. Figures denote degree of hemagglutination.

† Per cent of cells hemolyzed as determined colorimetrically.

The results of these experiments are presented in Table V. It is clear from them that the hemagglutinative and hemolytic activities of mumps virus are completely adsorbed on chicken erythrocytes at 4°C. and may subsequently

be released in part at least by elution at 37°C. Moreover, the cells from which the virus was eluted are no longer susceptible either to the hemagglutinative or hemolytic action of the virus. The cause of our failure to recover all the activity in the eluates is probably to be attributed to incomplete elution after the comparatively short interval of 2 hours.

Neutralization of the Hemolysin by Immune Serum

The action of mumps immune sera on the hemolysin was investigated.

Acute phase and convalescent sera were obtained from a patient with parotitis and a monkey in which the disease was produced experimentally. These sera, heated for $\frac{1}{2}$ hour at 56°C., were diluted in 0.5 cc. amounts of buffer (pH 7.2) containing diluted infected amniotic fluid (1:16) with a hemolytic titer of 1:128. The mixtures were placed at 4°C. overnight, and 0.5 cc. of 4 per cent suspension of red cells was added to each on the following morning. They

TABLE VI
Inhibition of the Hemolytic Activity of Mumps Virus by Immune Sera

Species	Serum	Dilutions of serum										Buffer control	Serum control
		8	16	32	64	128	256	512	1024	2048	4096		
Monkey	Normal	3*	5	23	23	27	27	27				29	0
	Convalescent	0	0	0	0	0	0	0	5	10	27	30	0
Man	Acute	8*	13	17	22	28	31					31	0
	Convalescent	0	0	4	7	13	16	15	33	33	34	31	0

* Per cent of cells hemolyzed determined colorimetrically with 4 per cent cells.

were then placed at 4°C. for 1 hour, shaken gently, and incubated for 4 hours at 37°C. As a control on the possible hemolytic action of the sera, dilutions of serum in buffer were prepared and an equal volume of 4 per cent red cells was added. These controls were treated in the same manner as the virus-serum-red cell mixtures. At the end of the incubation period, the degree of hemolysis was determined on the supernatant fluid by means of the photoelectric colorimeter.

The results are presented in Table VI. They show that the hemolysis of chicken red cells is inhibited by the sera of man and monkey convalescent from mumps. In contrast, normal monkey serum and serum taken during the early stage of the disease in man had only a slight inhibiting effect. These findings suggest that the hemolytic activity of amniotic fluid infected with mumps virus is due to a factor specifically produced by this agent.

Effect of Mumps Virus on the Fragility of Human and Chicken Erythrocytes

In view of the foregoing observations, it became of interest to see whether the adsorption of mumps virus on human and chicken cells would affect their fragility.

Red cells were recovered from defibrinated human blood. Chicken cells which had been recently collected in Alsever's solution were used. A 5 per cent solution of human albumin in saline was added to minimize the effect of handling on cell fragility. The cells were then washed three times in this mixture and a 6 per cent suspension of human or chicken red cells was prepared in the albumin-saline. To 40 ml. of the suspension of each of the two types of cells, 3 ml. of allantoic fluid infected with the virus of mumps was added. To serve as controls on the possible mechanical effects of hemagglutination, 3 ml. of PR8 and of Lee B influenza viruses were added to 40 ml. portions of these cell suspensions. To the same volumes of the suspensions of human and of chicken erythrocytes, 3 ml. of normal allantoic fluid was added to detect any non-specific action of this material. The virus-cell mixtures were allowed to stand at 25°C. for $\frac{1}{2}$ hour when the supernatant fluids were removed and tested for the presence of virus by means of the hemagglutination reaction. The results showed that almost all the mumps and influenza viruses had been adsorbed to the cells. The chicken cells were sedimented and then resuspended in fresh albumin-saline, whereas the human cells were resuspended in the original supernatant fluid during the subsequent elution at 37°C. for 1 hour. The eluates were then removed and the cells were washed twice in albumin-saline. Hemagglutination tests on the eluate showed that most of the virus in all instances had been released from the cells. Some of these cells were then shown to be agglutinated by the addition of fresh homologous virus. The residue of the cells was used to determine the osmotic fragility by methods which have been described (3).

Fig. 2 presents representative curves of the results. The data show that exposure to mumps virus caused a definite increase in fragility of human and chicken erythrocytes. On the other hand, adsorption and elution of influenza virus brought about no detectable effect. This latter finding is of especial significance in view of the fact that the influenza virus is known to inactivate the hemagglutinin receptors of the chicken red cell for mumps virus (5).

DISCUSSION

The observations on the hemolysin of the mumps virus which we have presented reveal a property exhibited by many bacteria but, in so far as we are aware, one hitherto undescribed among viruses. The experiments have been carried out with precautions to eliminate any hemolysis which might be caused by non-specific chemical or physical factors. This fact together with the findings that normal egg fluids or those obtained from embryos infected with PR8 or Lee B influenza viruses did not exert a hemolytic effect under the same conditions show that the hemolysin present in fluids infected with mumps virus is a specific product of this agent. From the results, two inferences have been drawn: (1) the hemolysin is not identical with the hemagglutinative property; (2) the hemolysin in its behavior is in many respects analogous to an enzyme.

Hemolytic activity can be experimentally distinguished from the hemagglutinative capacity of infected fluid by the inactivating effect of moderate heating, reaction temperature, and the pH range of activity. On the other hand, the two factors behave essentially alike in respect to their adsorption and elution with red cells and their inhibition by specific immune serum. It cannot therefore be now asserted that these two factors represent distinct constituents or

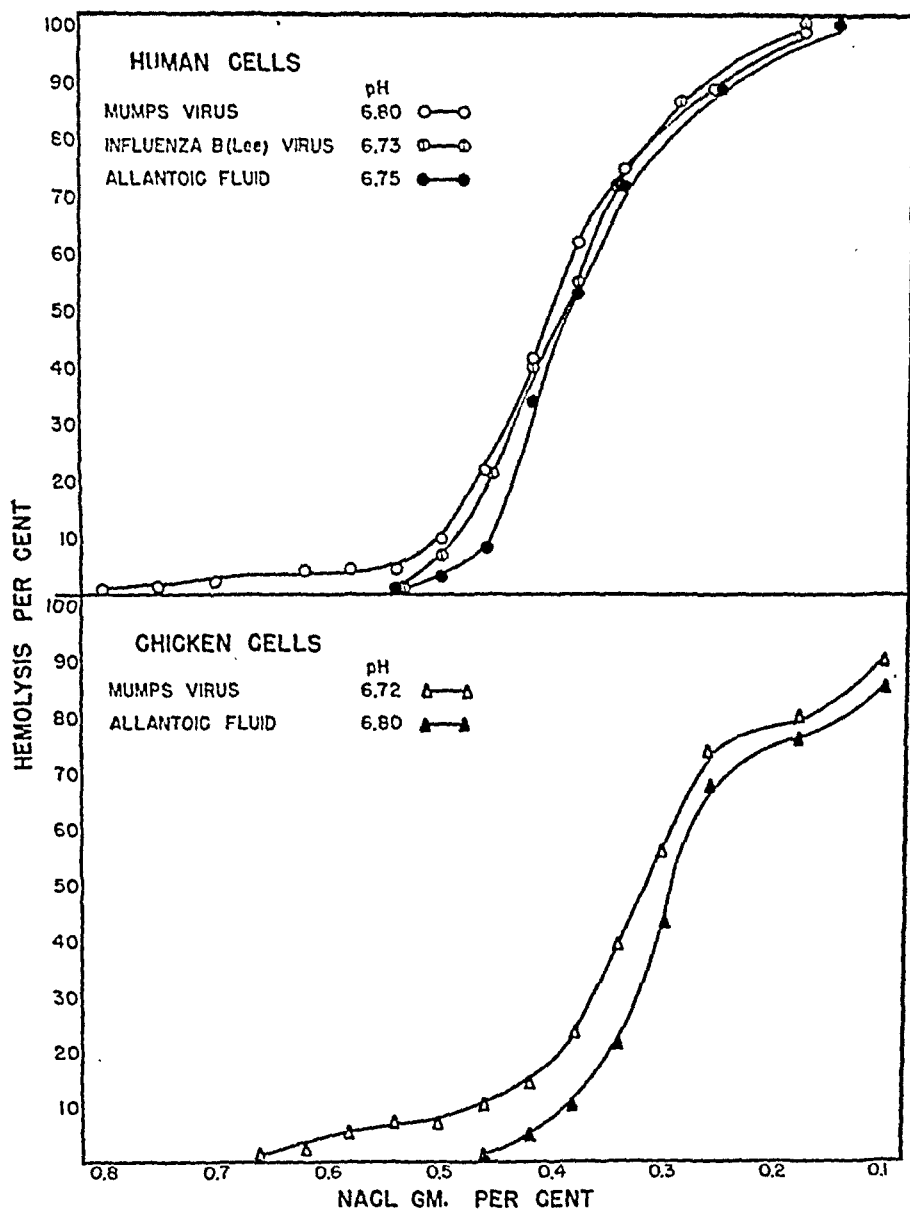


FIG. 2. Effect of the adsorption and elution of mumps and influenza (Lee B) virus on the osmotic fragility of human and chicken erythrocytes.

products of the virus. The determination of their exact relationship must await further experimentation carried out, for example, by differential ultrafiltration and centrifugation. In addition, studies to determine whether

the same cell receptor is involved in both reactions, might also contribute to the elucidation of this problem.² If through such experimentation, these two properties are shown to be manifestations of a single constituent, *i.e.* the infective particle, then it is possible that the concentration of hemolysin may afford a more accurate index of infectivity, for our findings indicate that the activity of the hemolysin is more easily impaired than that of the hemagglutinin.

Several features of the hemolysin resemble those of enzymes, such as inactivation by gentle heating, increase in activity correlated with the passage of time, increase of hemolytic effect within the temperature range of 4 to 37°C., the effect of hydrogen ion concentration on the degree of hemolysis. Furthermore, as shown by the results of elution experiments, the hemolysin is not exhausted during hemolysis but retains its characteristic activity when added to fresh cells. In this respect, of course, it behaves like the hemagglutinin. It may in this connection be pointed out that Burnet (6) has described an enzyme-like effect of influenza virus on gastric mucin and Bovarnick and de Burgh (7) have shown that this agent may inactivate comparatively large amounts of the red cell receptor substance without itself undergoing loss of activity.

SUMMARY

1. A factor capable of causing the hemolysis of the erythrocytes of man, chicken, and sheep occurs in the amniotic and allantoic fluids of chick embryos infected with the virus of mumps.

2. The hemolysin has not been found in normal fluids or in those infected with PR8 or Lee B strains of influenza virus.

3. The hemolysin is definitely inhibited by the serum of man and monkey convalescent from mumps, but only slightly by the serum of the acute phase.

4. The hemolysin is almost completely inactivated at 50°C. after 10 minutes. It exhibits maximal activity at 37°C. and is completely inactive at 4°C. A pH range from about 7.0 to 8.0 allows for maximum activity.

5. Adsorption and elution of the hemolysin with red blood cells has been demonstrated. After elution of the hemolysin, the red blood cells exhibit an increased osmotic fragility. Similar treatment of red cells with influenza virus did not alter this property.

6. The relationship of the hemolysin to the hemagglutinin and the enzyme-like behavior of the former have been discussed.

The authors gratefully acknowledge the technical assistance of Mrs. Virginia Peaslee.

² Since the completion of the experiments described in this paper, it has been determined that chicken erythrocytes, treated respectively with PR8 and Lee B influenza viruses in such a manner as to render them inagglutinable by mumps virus, were not hemolyzed by fluids infected with this agent. These fluids, however, exhibited marked hemolytic activity on suspensions of untreated cells. This finding might be interpreted as indicating fixation of the mumps hemagglutinin and hemolysin by the same cell receptor.

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THE RELATION OF INFECTIOUS AND HEMAGGLUTINATION TITERS TO THE ADAPTATION OF INFLUENZA VIRUS TO MICE*

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A number of serial passages is necessary before recently isolated strains of influenza virus can be adapted to mice. Little is known of the fundamental mechanism involved in the process of adaptation of the virus to mice. Hirst (1) reported that egg-adapted influenza A virus (Ala. 41, Kil. 41, N. Y. 43) while inducing no pulmonary lesions in mice, multiplied to the same maximum egg-infectious titer as the mouse-adapted passage of the same virus. The materials used by Hirst consisted of mouse lung preparations harvested 3 or 4 days after the virus was introduced intranasally. Thus the results, if correct, represent only the conditions occurring at the peak of virus growth. Since the adaptability of a virus may be more closely related to events taking place in the early stage rather than in the later stage of its growth, it was of interest to determine the earlier life history of the virus in mice. The present study is a report of observations on the rate of growth and the infectious titer of unadapted and adapted lines of a strain of influenza type A prime virus in the lungs of mice at various time intervals after intranasal introduction.

Methods

The influenza viruses used in the present experiments were two lines of the Rhodes strain (2), one adapted and the other unadapted to mice although both are propagated readily to approximately the same titers in the chorio-allantois of embryonated eggs. Antigenically they are the same, but their adaptability to mice is entirely different, one being pathogenic (adapted), and the other non-pathogenic (unadapted). At the time this study was instituted, the adapted line had been subjected to four ferret and fourteen mouse passages, and the unadapted line had been subjected to three ferret, two amniotic, and four allantoic passages.

Fresh preparations of equivalent concentrations of unadapted Rhodes (in the form of allantoic fluid) and adapted Rhodes (in the form of mouse lung suspension) virus were each inoculated intranasally (0.05 ml.) into similar groups of 20 to 24 young Swiss mice. The mice used in each experiment were of the same breed and of about the same size. Four mice from each similar group were sacrificed at 0, 4, 12, 24, 48, and 72 hour intervals after inoculation, and the lungs thus obtained at each interval from each group were ground and sus-

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pended in 10 per cent normal horse serum-saline. These mouse lung suspensions were centrifuged at 2000 R.P.M. for 5 minutes to remove the gross sediments. Tenfold dilutions of the fresh mouse lung suspensions were made in broth and 0.1 ml. amounts were inoculated into the allantoic sacs of three 10-day embryonated eggs. The eggs were incubated (35°C.) for 48 hours and then chilled at 4°C. overnight. The allantoic fluids were then tested individually

TABLE I

Rate of Multiplication of Unadapted and Adapted Lines of Rhodes Virus in Mouse Lung

Experiment No.	Rhodes viruses	Mice used	Dilution	Volume inoculated	Route of inoculation	Method of titrating virus	Titer at intervals in hours after I.N. inoculations*					
							0	4	12	24	48	72
I	Unadapted	20				E. I. ₅₀ Hemagglutinin	0 0	10 ^{-0.5} 0	10 ^{-3.4} 0	10 ^{-3.5} <20†	10 ^{-6.5} <20†	N.D. "
	Adapted	20	10 ⁻²	0.05	I.N.	E. I. ₅₀ Hemagglutinin	10 ^{-2.0} 0	10 ^{-1.5} 0	10 ^{-7.0} 0	10 ^{-7.5} 1,280	10 ^{-7.4} 1,280	" "
II	Unadapted	24				E. I. ₅₀ Hemagglutinin	10 ^{-2.0} 0	10 ^{-1.5} 0	10 ^{-3.5} 0	10 ^{-5.5} 0	10 ^{-6.5} <20†	10 ^{-6.7} 0
	Adapted	20	10 ⁻²	0.05	I.N.	E. I. ₅₀ Hemagglutinin	10 ^{-3.5} 0	10 ^{-4.0} 0	10 ^{-6.7} <20†	10 ^{-7.5} 1,280	10 ^{-7.5} 640	N.D. "

N.D., not done.

I.N., intranasal.

E.I.₅₀, 50 per cent egg-infectious titer.

* Lungs of four mice were pooled at each interval.

† Hemagglutination \pm at 1/20.

for hemagglutinating titer by a pattern method (3), and the 50 per cent egg infectious titer calculated (4). The mouse lung suspensions at each time interval were also tested for hemagglutinating titers.

EXPERIMENTAL

Two separate experiments were conducted by similar procedures as described. The data are recorded in Table I and Figs. 1 and 2.

It will be observed that the growth of the line of unadapted Rhodes in mice is much slower than that of the line of adapted Rhodes. At 4 and 12 hours

after inoculation, the egg-infectious titer ($E.I._{50}$) of mouse lung suspensions of the unadapted line is low ($10^{-3.8}$ or less), and the peak of growth is not reached until 48 hours. On the other hand, the adapted line multiplied rapidly after its introduction into mice, and nearly attained its peak of growth within 12 hours ($E.I._{50}$: $10^{-6.7}$ to $10^{-7.0}$). The latter finding is in agreement with Taylor (5) who found that the PR8 strain of influenza virus reached a maximum M.L.D. titer in mouse lungs within 24 hours after intranasal introduction. In

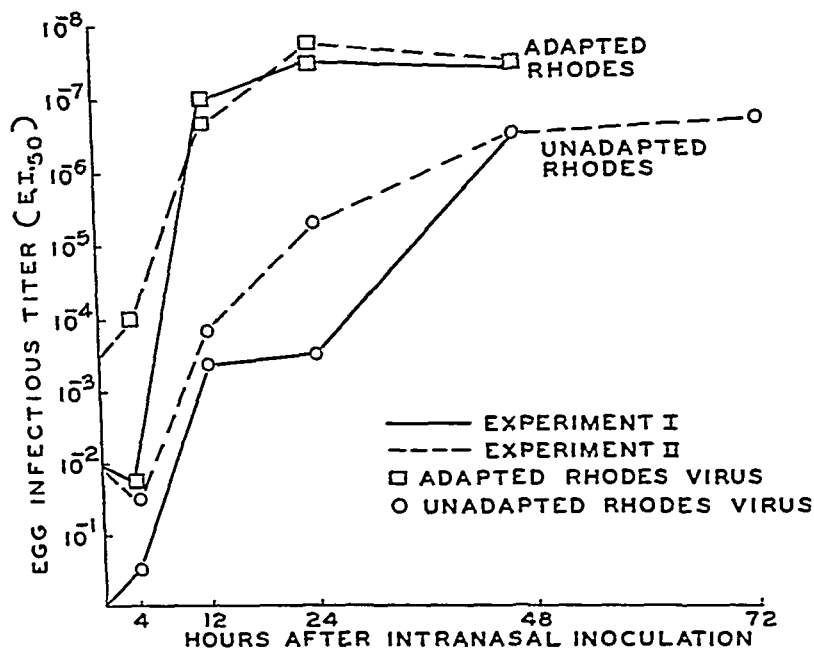


FIG. 1. Rate of multiplication of mouse-adapted and unadapted lines of Rhodes virus in mouse lung expressed as 50 per cent end-point infectious titer in eggs.

addition, the hemagglutination titers obtained from the multiplication of these two lines of virus in mouse lung are also different. This is clearly shown in Fig. 2. The hemagglutination produced by the unadapted line is exceedingly low (less than 20) even when the virus has reached its peak of growth at the end of 48 or 72 hours, while the adapted line gives a titer of 1,280 as early as 24 hours after inoculation. Since hemagglutinin is not readily separable from the influenza virus particles (6, 7), it would tend to indicate that there have been more virus particles produced by the adapted line than by the unadapted line at the peak of growth in the pulmonary tissue of mice.

DISCUSSION

It is of considerable interest to note that the line of mouse-unadapted Rhodes strain (ferret-egg passage), though completely non-pathogenic to mice, can

multiply readily in the pulmonary tissue of these animals. This finding is in agreement with that of Hirst (1). In contrast to Hirst's results, however, was the fact that the titer of virus concentration of the unadapted line was much lower than that of the adapted line (ferret-mouse passage). Hirst reported that the mouse-unadapted and adapted passages of influenza virus multiplied to the same maximum titer in the pulmonary tissue of mice. The discrepancy may be accounted for by the facts that (a) Hirst did not sacrifice his animals until 3 or 4 days after virus inoculation while mice were sacrificed at repeated

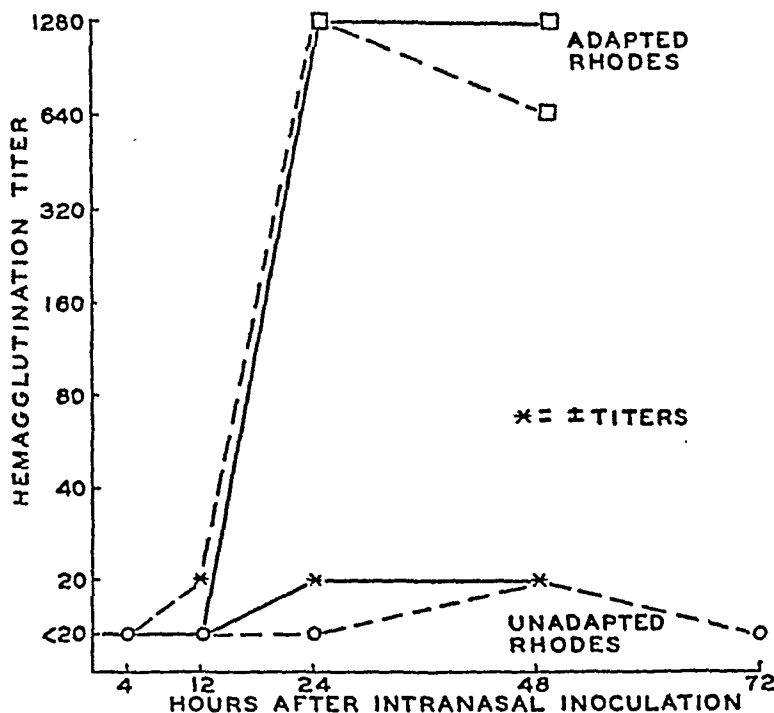


FIG. 2. Hemagglutination titers of the same mouse lung virus preparations as in Fig. 1

intervals in the present study; (b) a comparison of different passages of the same strain was made by Hirst while two different lines (unadapted and adapted) of the Rhodes strain in simultaneous passage were used in the present experiments. Whatever the explanation, the fact remains that the mouse-adapted line reaches a much higher hemagglutination titer (1,280) at the peak of its growth than does the unadapted line (less than 20). Since hemagglutination is intimately associated with the particles of influenza virus (6, 7), a higher hemagglutinating titer would logically imply a higher virus titer. It is to be pointed out, however, that the egg-infectious titers attained by these two lines of virus at the peak of their growth are not markedly different, $10^{-7.5}$ to

$10^{-7.8}$ for the adapted, *versus* $10^{-6.5}$ for the unadapted line. The discrepancy between egg-infectious and hemagglutination titers was also observed with the adapted strain in eggs after 12 and 48 hours of incubation ($35^{\circ}\text{C}.$). The egg infectivity closely approached the peak at 12 hours ($10^{-6.7}$ to $10^{-7.0}$) when no hemagglutination was detectable, while another 12 hours of incubation raised the hemagglutination titer from less than 20 to 1,280 without appreciably increasing the egg-infectious titer.

It seems that the virus particles in mouse lungs infected with the adapted line present two characteristics: one being infective for eggs, and the other being capable of agglutinating chicken red cells in high titer. The line unadapted for mice on the other hand, produced a pattern of high egg infectivity and low hemagglutinating titer. This tends to indicate that the capacity of the virus to agglutinate chicken red cells to high dilution is a better measure of its pathogenicity for mice than the egg-infectious capacity.

It is also significant to note that the mouse-pathogenic virus (adapted Rhodes) had a much more rapid growth rate in mice than that of the non-pathogenic line. This difference in growth rates might well explain why some variants of one virus may easily be adapted to mice while with others greater difficulty is encountered. The adaptation of a virus to mice or to other animals through passages might be the result of an actual natural selection of the more rapidly growing variants of the same virus.

SUMMARY

The non-pathogenic (unadapted) line of the Rhodes strain of influenza virus multiplied readily in the mouse lung, but its rate of multiplication was slower and its hemagglutination titer attained at the peak of growth was much lower than that of the adapted line of the same virus. The implication of these findings to mouse adaptability of the virus is discussed.

The author wishes to express his deep appreciation to Dr. Thomas Francis, Jr., for his direction, encouragement, and advice in making this work possible.

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SPECIFIC SERUM AGGLUTINATION OF ERYTHROCYTES SENSITIZED WITH EXTRACTS OF TUBERCLE BACILLI

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It is known that red blood corpuscles can adsorb various substances and thereby be rendered specifically agglutinable by serum antibody directed against the substance adsorbed (1-4). The present paper describes observations on the agglutination, by the sera of experimental animals and of tuberculous patients, of erythrocytes previously treated with aqueous extracts of tubercle bacilli or with products of their culture filtrate.

Materials and Methods

Preparation of Extracts of Tubercle Bacilli.—The H37Rv strain of human tubercle bacillus was cultivated on the surface of a liquid medium described in a previous communication (medium 7 in reference 5). After approximately 14 days of growth, the whole bacillary mass was filtered free of the culture filtrate and washed on the filter, first with distilled water, then with cold acetone. Following removal from the filter, the organisms were air-dried at room temperature in a glass container. All these operations were carried out under irradiation with ultraviolet light. The dried bacillary material was stored at room temperature or in a refrigerator.

Four grams of air-dried bacilli was suspended in 100 cc. of 88 per cent phenol (phenol liquefied, U. S. P. XIII, Mallinckrodt) and stirred by a magnetic stirrer at 35°C. for 20 hours; the suspension was centrifuged and the clear brownish supernatant fluid discarded. The sediment was resuspended in 100 cc. of fresh phenol solution and the same operation was repeated. The new sediment was twice resuspended in fresh phenol solution, stirred for only $\frac{1}{2}$ hour each time, and centrifuged. It was then washed three times with 90 cc. aliquots of cold acetone in order to remove the phenol. Finally, the phenol-acetone-treated bacillary mass was dried and stored at room temperature.

The phenol-acetone-treated, dried bacillary material (0.5 gm.) was suspended with mortar and pestle in an isotonic aqueous solution (65 cc.) containing 0.55 per cent NaCl, 0.5 per cent Na_2HPO_4 , and 20 per cent methanol. The methanol served to facilitate suspension of the bacilli as well as to prevent contamination. This suspension was stirred for 20 hours by a magnetic stirrer at 35°C. The extract was freed of most of the suspended bacillary bodies by centrifugation in an angle head centrifuge (Sorval type) at 10,000 R.P.M. for 30 minutes. The slightly opalescent extract was adjusted to a pH of 6.0, was dialyzed through ordinary sausage casing cellophane against tap water for 6 hours, and then against distilled water for 1 to 3 days. The solution in the cellophane bag was evaporated down to a volume of 20 cc. in front of a fan, removed from the bag, and centrifuged at high speed to yield an almost clear extract which was adjusted to pH 6.5 to 7.0 with dilute NaOH and made isotonic by the addition of 0.52 cc. of saturated aqueous solution of sodium chloride.

Preparation of Washed, Packed, Sheep Erythrocytes.—Sheep's blood was collected aseptically in 1.2 volumes of sterile modified Alsever's solution (6). The red cells in this mixture remain adequate for the hemagglutination test for 3 months when kept under aseptic conditions at

4°C. The mixture of blood and Alsever's solution was centrifuged and the red cells were washed three times with 6 volumes of isotonic saline solution. After the last centrifugation at 2000 R.P.M. for 20 minutes, as much of the supernatant fluid as possible was removed and the packed cells were stored at 4°C.; these cells were used within 3 days or until hemolysis was clearly evident. Fifty cc. of blood-Alsever's mixture yielded about 7 cc. of packed, washed erythrocytes.

Sensitization of Sheep's Cells with Extract.—Approximately 0.5 cc. of packed, washed red cells was added to 10 cc. of the neutral, isotonic extract of tubercle bacilli; the cells were well suspended and placed in a water bath at 37°C. for 2 hours and frequently agitated; then the suspension was centrifuged and the clear supernatant fluid was discarded. The packed treated red cells were washed three times with 30 cc. of saline and finally suspended in 100 cc. of sterile saline (0.5 per cent "sensitized red cell suspension") to be stored at 4°C. and used within 3 days or until hemolysis was clearly evident.

Collection and Preparation of Serum for Absorption.—Blood was collected and allowed to clot, and the clot allowed to retract; serum was removed and, for preservation, when desired, merthiolate was added in a final concentration of 0.01 per cent. To 1 cc. of serum was added 1 cc. of saline and this mixture was heated at 56°C. for 30 minutes in order to inactivate complement.

Absorption of Serum with Untreated Red Cells.—The following operations were designed to remove from the sera, antibodies unrelated to tuberculous infection which would agglutinate untreated red cells by the hemagglutination technique to be described.

In 2 cc. of 1:2 dilution of heated serum 0.2 cc. of packed, washed, untreated sheep's cells was suspended, and the suspension was allowed to stand at room temperature for at least 20 minutes, during which it was frequently shaken. It was then centrifuged to sediment the cells and the supernatant fluid was removed and treated in the same way with another 0.2 cc. of packed untreated cells. The final supernatant fluid was used in the hemagglutination tests as representing a 1:2 dilution of absorbed serum.

A 0.5 per cent suspension of untreated red cells, to be used as control antigen in the testing of all sera, was prepared by suspending 0.1 cc. of untreated, washed, packed sheep cells in 20 cc. of isotonic sterile saline. This suspension was stored in the refrigerator and used no longer than 3 days after its preparation.

Performance of Hemagglutination Test.—Twofold serial dilutions of the absorbed serum to be tested were made in saline in tubes of 12 mm. internal diameter. To tubes containing 0.4 cc. of serum dilution there was added 0.4 cc. of 0.5 per cent suspension of treated erythrocytes. Two control tubes were found to be necessary: one containing 0.4 cc. of saline and 0.4 cc. of 0.5 per cent suspension of treated cells, and the other containing 0.4 cc. of 1:2 dilution of absorbed serum and 0.4 cc. of 0.5 per cent suspension of untreated erythrocytes.

Incubation at 37°C. for 2 hours, followed by a preliminary observation, a vigorous shaking, and further incubation at room temperature overnight, was found to allow satisfactory reading of the degree of agglutination. The results were read and recorded as in any red cell agglutination test.

Agglutination of the treated cells in saline has usually been present to an insignificant extent and this has served as a guide in the judging of clear cut agglutination in an actively agglutinating serum. Agglutination of untreated red cells by the absorbed serum indicates that the serum has been incompletely absorbed and renders the test invalid.

Technique of Inhibition of Specific Hemagglutination by Solutions Containing Sensitizing Material.—An active serum was selected which agglutinated treated red cells at high titre, and a serum dilution near the end-point of agglutination was carefully determined which consistently gave definite, though not strong, agglutination of treated red cells. Serum in this dilution was added in 0.4 cc. amounts to a series of tubes containing 0.4 cc. of serial dilu-

tions of the substance to be tested for its ability to inhibit the specific agglutination. The tubes were incubated in a water bath at 37°C. for 45 minutes. To each tube was then added 0.4 cc. of a 0.5 per cent suspension of treated red cells. Incubation and the reading of results were carried out as in the case of the above described hemagglutination test. Appropriate controls containing untreated red cells with the undiluted solution to be tested, treated cells with saline, and treated cells with serum, respectively, were included in such tests.

EXPERIMENTAL RESULTS

Study of Sera.—Table I presents the results obtained in the examination of various active sera.

TABLE I
Agglutination of Sensitized Red Cells by the Sera of Rabbits Injected with BCG

Dilutions of sera in saline	Immune rabbit sera						Normal rabbit serum
	1 (742)	2 (744)	3 (745)	4 (734)	5 (735)	6 (735)	
1:10	++++	++++	++++	++++	++++	++++	—
1:20	++++	++++	++++	++++	++++	++++	—
1:40	++++	++++	++	++++	++++	++++	—
1:80	++++	++++	+	++++	++++	++++	—
1:160	+++	+++	—	++++	++++	++++	—
1:320	+	+	—	++++	++++	++++	—
1:640	—	—	—	+++	+++	++	—
1:1280	—	—	—	++	+	+	—
1:2560	—	—	—	—	—	—	—
1:10 with untreated red cells	—	—	—	—	—	—	—

Sera 1, 2, and 3 were drawn from rabbits which had received 8 weeks previously one single intravenous injection of 0.2 mg. of a living culture of a strain of BCG identified as BCG 317 (7). Sera 4, 5, and 6 were drawn from rabbits injected according to a different schedule: the animals were injected by the intravenous route with 0.2 mg. of a living culture of the same strain of BCG and were allowed to rest for 5 weeks; then three weekly injections of 0.02 mg. of the same culture in 1 cc. of saline were made into the left heart in order to distribute the bacilli into parts of the body other than the lung.

The data presented show that the titres of antibody against the treated red cells were consistently and significantly higher in the rabbits repeatedly injected with tubercle bacilli.

In Table II are recorded the results obtained with the sera of some patients with active pulmonary tuberculosis. No correlation has yet been attempted between the degree of activity of the disease and the titre of the serum in the hemagglutination test, but it is evident that all the patients tested had titres

of 1:8 or higher; that is to say, they possessed antibodies capable of agglutinating the sensitized red cells. One additional serum from a patient with miliary tuberculosis, made available to us by Dr. Walsh McDermott of the New York Hospital, had a titre of 1:256.

Some evidence has been obtained for the specificity of the hemagglutination reaction by testing red cells treated with the extract of tubercle bacilli against the sera of experimental animals which had been immunized with other micro-organisms:¹ *Pneumococcus* Type I, *Pneumococcus* Type III, *Pneumococcus* Type XIV, Friedländer bacilli types B and C, and Flexner dysentery bacilli types X and Y. There were weak cross-reactions with Friedländer antisera which gave doubtful agglutination at 1:2 dilutions of serum; these reactions can be considered insignificant. There were also weak reactions with high

TABLE II
Agglutination of Sensitized Red Cells by the Sera of Tuberculous Patients

Dilutions of sera in saline	Human sera						Saline control
	1	2	3	4	5	6	
1:2	+++	++++	+++	++++	++++	++++	—
1:4	++	++++	++	++++	++++	++++	—
1:8	+	++++	+	++++	+++	++++	—
1:16	—	++	±	+++	+++	++	—
1:32	—	±	—	++	++	+	—
1:64	—	—	—	+	±	—	—
1:2 with untreated red cells	—	—	—	—	—	—	—

titled *Pneumococcus* Type XIV antisera, both from the horse and from the rabbit; these were not marked, and appeared only in the dilution below 1:32.

Table III gives further evidence for the specificity of the reaction. Serum 1 was a pool of sera from 10 to 20 individuals all giving strongly positive Wassermann reactions for syphilis;² it is probable that many of these individuals were tuberculin-positive, but there was no evidence that any of them had active tuberculous disease. The pooled serum was also investigated for its ability, at a dilution of 1:4, to inhibit specific hemagglutination by a selected active serum, but there was no evidence of such inhibitory activity. Sera 2, 3, 4, and 5 were from patients with or convalescing from acute streptococcal infections.³

¹ Kindly supplied for our use by Dr. Walther Goebel and Dr. Frank Horsfall of the Rockefeller Institute.

² This pool was generously supplied by Dr. Widelock of the Serology Laboratories of the New York City Department of Health.

³ Made available through the kindness of Dr. Sidney Rothbard of the Hospital of the Rockefeller Institute.

Sera 6, 7, 8, 9, and 10 were from tuberculin-negative student nurses at the New York Hospital.⁴ Serum 11 was from a tuberculin-positive individual who had been working for many months with living tubercle bacilli but had no evidence of active disease. As will be noted from the results presented in Table III, none of these sera gave titres higher than 1:4 in the hemagglutination test.

Study of Antigen.—Material from the tubercle bacillus which sensitizes red cells to agglutination in tuberculous antisera has been demonstrated to be present in at least one preparation of old tuberculin. Thus, one sample of Gilliland O. T. (Wyeth), which had been steamed for many hours during preparation, was observed to be as effective in sensitizing sheep erythrocytes as the extract described here. One ml. of this deglycerinated O. T.⁵ was capable

TABLE III
Agglutination of Sensitized Red Cells by the Sera of Non-Tuberculous Individuals

Dilutions of sera in saline	Human sera											Saline control
	1	2	3	4	5	6	7	8	9	10	11	
1:2	±	—	—	—	—	++	—	—	—	++	+	—
1:4	—	—	—	—	—	±	—	—	—	+	—	—
1:8	—	—	—	—	—	—	—	—	—	±	—	—
1:16	—	—	—	—	—	—	—	—	—	—	—	—
1:32	—	—	—	—	—	—	—	—	—	—	—	—
1:2 with untreated red cells	—	—	—	—	—	—	—	—	—	—	—	—

of sensitizing completely 0.025 cc. of washed packed sheep erythrocytes to such an extent that they were agglutinated in the same way and to the same titre in an anti-BCG hyperimmune serum (No. 734) as were the cells of the same lot sensitized by the above described extract of tubercle bacilli. This fact shows that at least one substance responsible for sensitization is heat-stable and is present in the culture filtrate of cultures of mammalian tubercle bacilli.

Additional experiments were performed in an attempt to determine whether active material was present in the carbohydrate or in the protein fraction of the bacillary extracts and culture filtrates. Serial dilutions of these fractions were made in saline and were tested for their relative ability to inhibit specific hemagglutination of red cells sensitized by the extract of H37Rv. Table IV reveals the results of these experiments. Extract fraction 1 was the crude aqueous extract described in this paper. Extract fraction 2 was an aliquot of

⁴ Made available through the kindness of Dr. F. Lansdown and Dr. C. Muschenheim of the New York Hospital.

⁵ Available through the courtesy of Dr. Merrill Chase of the Rockefeller Institute.

the same extract from which most of the protein had been removed by acidification with dilute HCl and repeated shaking with chloroform and isoamyl alcohol (8) until there was very little material at the chloroform-water interphase after centrifugation. It is evident that this procedure failed to decrease significantly the ability of the extract to inhibit specific hemagglutination even by a low dilution of antiserum.

TABLE IV
Inhibition of Specific Hemagglutination by Extracts and Fractions of Tubercle Bacilli

Dilutions of extracts in saline	Extracts and fractions							
	1*	2*	1†	2†	1§	2§	3§	4§
Undiluted	—	—	—	—	—	—	—	—
1:2	—	—	—	—	—	—	—	—
1:4	—	—	—	—	—	—	—	—
1:8	—	—	—	—	—	—	+	—
1:16	—	—	—	—	—	—	++	—
1:32	—	+	—	—	—	—	++	±
1:64	++	+++	—	—	—	—	++	+
1:128	+++	+++	—	—	—	—	++	++
1:256			—	—	—	—	++	++
1:512			—	—	—	—	++	++
1:1024			—	—	+	+	++	++
1:2048			—	—	++	++	++	++
1:4096			±	+				
1:8192			++	++				
Saline control	+++	+++	++	++	++	++	++	++

* Tested against 1:10 dilution of BCG rabbit antiserum 735, absorbed with untreated red cells.

† Tested against 1:320 dilution of BCG rabbit antiserum 735, absorbed with untreated red cells.

§ Tested against 1:320 dilution of BCG rabbit antiserum 734, absorbed with untreated red cells.

Unheated culture filtrate fractions 3 and 4, made available for our use by Dr. Janet McCarter Woolley, had been defined chemically as follows. Fraction 3 was known to contain only very little polysaccharide and approximately 20 γ /0.4 cc. of tuberculoprotein as determined by non-nucleic acid nitrogen content. Fraction 4, on the contrary, contained approximately 20 γ /0.4 cc. of tuberculopolysaccharide and no more than one part of tuberculoprotein to 19 parts of polysaccharide. It is evident that material active in the hemagglutination test was in the polysaccharide fraction and that amounts as small as 0.16 γ /0.4 cc. of solution could be detected by the technique of specific inhibition

of hemagglutination. It is clear, also, that it is possible by these techniques to standardize a solution of any extract or product of the culture filtrates of tubercle bacilli, with respect to activity in sensitizing sheep erythrocytes for the specific hemagglutination test.

The Use of an Avirulent Culture of Tubercle Bacilli for the Preparation of Sensitized Sheep Erythrocytes.—The avirulent variant, H37Ra (8), of the virulent strain, H37Rv, has been investigated for its ability to yield material capable of sensitizing sheep erythrocytes and to inhibit the specific hemagglutination of erythrocytes treated with the extract of H37Rv. No significant differences of a qualitative nature have, thus far, been detected in these respects between the two variant strains by the use of the high titred experimental animal sera or of sera of tuberculous patients.

DISCUSSION

The observations described show that at least one heat-stable component present in a polysaccharide fraction of the tubercle bacillus, can be adsorbed onto sheep erythrocytes, rendering them specifically agglutinable by antibody directed against the adsorbed material. The antibody responsible for this hemagglutination test circulates in the blood of immunized animals and of human beings with active tuberculosis. The test exhibits a high degree of specificity and, in particular, does not give rise to any cross-reaction with Wassermann-positive sera as sometimes occurs in the case of the complement fixation reactions in tuberculosis (9).

The specific hemagglutination reaction can be inhibited by adding the soluble reactive antigen to the serum before introducing the sensitized red cells into the system. Under these circumstances the soluble antigen, if present in sufficient amount, combines with its corresponding antibody and prevents it from agglutinating the sensitized erythrocytes. Thus this inhibition test, when utilized together with the agglutination test proper, permits the detection and quantitation of very small amounts of the sensitizing antigen, as repeated observations have shown. It will be interesting to test whether this technique permits the detection of specific antigen circulating *in vivo*.

SUMMARY

A hemagglutination reaction has been described between sheep erythrocytes treated with a component of a polysaccharide fraction of mammalian tubercle bacilli and the sera of experimental animals or of tuberculous patients.

Evidence has been presented for the specificity of this reaction. A modification of the test, involving an inhibition reaction, has been developed for the detection and quantitation of minute amounts of the material responsible for the hemagglutination reaction.

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THE NEOPLASTIC POTENTIALITIES OF MOUSE EMBRYO TISSUES

IV. LUNG ADENOMAS IN BABY MICE AS RESULT OF PRENATAL EXPOSURE TO URETHANE*

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PLATES 24 TO 28

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In previous papers from this laboratory (1) experiments have been described which were made to learn how soon in the life of the organism cells possess the ability to become tumor cells. The neoplastic potentialities of various mouse embryo tissues, procured in the latter half of gestation, were tested by transplanting them to adults, together with methylcholanthrene. Tumors arose swiftly and in great diversity, yet it was questionable whether the cells exposed to the carcinogen were still in the embryonic state when they underwent neoplastic change, since the interval before the growths first became perceptible somewhat exceeded the time until birth, had the embryos been left undisturbed. If it had been possible to utilize the cells of very young embryos perhaps tumors could have been obtained within this period, but they did not survive the requisite exposure to methylcholanthrene, even when this was circumspectly injected into the "beads" along the uterus. Obviously for a decisive test of the neoplastic potentialities of embryo cells these must be exposed *in utero* to a carcinogen acting so speedily that its neoplastic effects will be evident almost at once. Recent authors who have produced pathological changes in mouse embryos with the Roentgen rays (2) have made no mention of tumor formation, and the polycyclic hydrocarbons fail to pass the placenta in effective quantity. It was recalled however that the injection of the highly diffusible hypnotic, urethane, into adult mice of strains liable to pulmonary adenomas in old age causes these growths to appear earlier than usual and in much greater number (3). We had employed such a strain of animals when testing with methylcholanthrene the neoplastic potentialities of transplanted fragments of embryo lung, and had noted that multiple adenomas formed within 2 to 3 weeks (4). Consequently urethane was now injected repeatedly into pregnant females, and their offspring were searched for adenomas. These were sometimes visible in 3-day-old animals, and often had attained a considerable size within 10 days, none appearing in controls. The urethane could have acted *in utero* for only a few hours, and it produced no visible damage in the lungs of

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the embryos exposed to it, which might have served as the basis for neoplastic changes occurring secondarily. All went to show that these changes were primary and took place prior to birth.

Law (5) has subjected embryonic tissues to the action of dibenzanthracene, injecting an olive oil solution of it directly into the amniotic sac of mouse embryos at the 15th day of gestation. The animals were killed when 200 days old on the average, and nearly all had lung tumors, in most cases multiple. While some of the growths may have been malignant the context of Law's paper indicates that the great majority were adenomas. The only other tumor developing was a fibrosarcoma of the skull in one instance.

Larsen has lately reported the presence of induced pulmonary adenomas in the 6-months-old offspring of A strain mice receiving urethane while pregnant (6). The work now described was done without knowledge of his. He found that urethane gave rise to few tumors in the young unless injected into the mother animals during the last 24 hours before parturition, and he noted that these latter showed many more growths than their young when all were examined together. His revealing observations will be considered in detail further on.

Materials and Methods

Mice of the C strain were mainly employed, as in the previous tests with transplanted embryo tissue. A large colony of the animals was available. In corollary several experiments were carried out with A mice raised in our laboratory.

The number of adenomas induced by urethane in adult mice is directly proportionate to the amount of the substance given (7). Hence it was injected into most of the pregnant females on several successive days, to a maximum of six, and in the greatest tolerable quantity. The precise duration of pregnancy was determined by the vaginal plug method in many instances, but palpation was relied upon in the majority to gain an idea of the age of the embryos. The injections were frequently begun as soon as these had pulmonary tissue for the urethane to act upon, that is to say round about the 12th day of gestation,¹ since our aim was to induce neoplastic changes as early as possible. Most of the females injected at that time either died or aborted as the injections were repeated, and our data have been procured in the main from those nearer term. Adult mice are known to differ widely in their tolerance of urethane.

At each injection the pregnant animal received, irrespective of its weight, 0.3 cc. of a 10 per cent solution of urethane in the subcutaneous tissue of the back. This produced 1 to 3 hours of apparent unconsciousness. The solution was very hypertonic because through a technical lapse the urethane had been dissolved in 0.9 per cent salt solution. Water bottles were used to supplement the fluid obtained from the diet of bread, milk, and lettuce. Not a few of the young born after the usual 21 days of gestation were puny and far below the normal weight, and these got their hair late and grew but slowly during several weeks. Ultimately

¹ We have been unable to find any account of when the lungs begin to form in the mouse. They are recognizable in embryos 12 days old. The tracheal anlage can then be seen pushing out from the foregut and ballooning into lung buds with primitive bronchi. It would be difficult to identify the lungs previous to this stage.

many litters were obtained however which resembled most of the controls in weight and vigor.

Some of the injected females were killed on the 18th to 20th day of gestation, and others on the 20th or 21st day, or when delivery had begun, in order to procure lungs which had not been exposed to postnatal influences. If the fetuses failed to breathe after removal from the uterus respiration was stimulated by dropping them repeatedly from a height of a few centimeters; and after they had taken a few breaths the chest was opened widely by lateral cuts, a flap including the sternum was everted, and the whole creature was plunged into acid Zenker's solution, for fixation of the pulmonary tissue *in situ*. Day-old mice were chilled in an ice box until insensible, and their lungs were fixed by the same procedure. Three-day- and 10-day-old young were killed by pushing in the cranium, after which a cord, already placed around the neck, was quickly tightened to prevent any aspiration of blood. The lungs were then dissected out and fixed as usual. By these procedures well distended pulmonary tissue was obtained, in which tiny adenomas could readily be perceived with the microscope. Mice 15 days old or older were chloroformed and the lungs were allowed to collapse to some extent prior to fixation, in order to reduce the number of sections to be examined. When the litters were large some of their members were killed 1 or 3 days after birth, in order to render the conditions more favorable to development of adenomas in the others; for the occurrence of these growths in adult mice is largely dependent upon sufficient food (8). Three groups of young animals, 3 days, 10 days, and 60 to 70 days old respectively, were searched intensively for adenomas, together with the appropriate controls. Many of the mother animals were killed and examined at the same time as their last surviving young.

Not a few of the 60 to 70-day-old offspring of urethanized mice had adenomas visible in the gross on the pleural surface, whereas none of the controls had any. They could not be seen on the pleura of 10-day-old animals, even at a magnification of $\times 17$, and hence the lungs of these and of 3-day- and 1-day-old individuals were searched *in toto* in serial sections 7.5μ thick, as were also those of fetuses near term. The pulmonary tissue of certain of the older animals was searched in the same way, notably when the incidence of tumors in mothers and young was to be compared. The searching was done, section by section, at a magnification of 120 diameters in the case of mice 10 to 70 days old, and at 180 diameters in that of younger creatures. This serial scrutiny proved crucial in the case of adenomas just forming, because they were often simulated in individual sections by cell groups cut through at the bend of a blood vessel or bronchiole, or were mimicked by pleural infoldings.

The choice of stains, methylene blue and eosin, proved fortunate, for they made the tiny adenomas of young animals stand out from their surroundings, ordinary lung parenchyma appearing purplish-pink save for the nuclei, whereas the adenoma cells were basophilic, sky blue to dark blue, according to the intensity of the staining.

Findings in the 60 to 70-Day-Old Offspring of Urethanized Mothers

Pure breeds of mice differ widely in their liability to spontaneous adenomas. These appear soonest and are most frequent in animals of the A strain, the C strain ranking next but far behind. Andervont found them in 20 to 30 per cent of C mice over a year old (9), as nodules visible in the gross on the pleural surface.—the "peripheral index"; but in our colony they almost never manifest themselves in this way in individuals less than 9 months old, much older ones often showing none. They are nearly always solitary. No one has tested the response of C animals to urethane, but to methylcholanthrene it is notably slow. The tumors were still absent from Shimkin's animals 13

weeks after an intravenous injection of this hydrocarbon, and there were only 4 to a mouse after 20 weeks, whereas in adults of the A strain they averaged 25 to an animal after 6 weeks, 31 after 13 weeks, and 47 after 20 weeks (10).

These findings gave no ground for the supposition that urethane would produce adenomas swiftly in the offspring of urethanized C mice; only their presence in implants of embryo tissue which had been acted upon by methylcholanthrene made early examination of the young seem worth while. In serial sections of the lungs of a 30-day-old animal of the first litter from a urethanized mother a typical adenoma was encountered, and in the other mouse of this litter, killed 60 days after birth, there was a growth on the pleural surface visible to the unaided eye. Consequently we now set aside many newborn mice and examined them in the gross 60 to 70 days later. The controls were in general killed when slightly older, mostly after 70 to 100 days, which tipped the scales somewhat in their favor because the longer a mouse lives the greater is the likelihood that adenomas will appear spontaneously. In a total of 97 controls examined when 60 to 104 days old, no tumor was to be seen on the pleural surface, whereas adenomas were present in 21 of 80 individuals 60 to 70 days old from urethanized mothers,—just such tumors both in the gross and microscopically, as develop spontaneously in adults. They averaged a millimeter in diameter, but a few were as much as 2 mm. across, and in occasional individuals two or three of them were present. The lungs were remarkably free from equivocal nodules.

In one young animal out of a group of seven killed when 30 days old, from four litters born to urethanized females, an adenoma had developed that was visible in the gross. Its character was confirmed with the microscope.

The Growths in Mice 10 Days and 3 Days Old

Adenomas proved fairly frequent on microscopic examination of the lungs of 15-day-old animals from urethanized mothers, and hence an intensive search was made for them in serial sections from still younger individuals. Tables 1 and 2 give the findings in groups 10 days and 3 days old respectively. It will be seen that 10 out of 16 of the 10-day-old sucklings had adenomas, in some cases two or three. In none of the lungs from 16 control animals 10 days old was any structure found that could be classed as an adenoma with certainty, though the organs were searched in serial sections with equal care.

Only characteristic adenomas (Figs. 1-3) have been given place in Table 1, of 10-day sucklings. Some were surprisingly large, as much as 0.2 mm. across, and not infrequently compression of the adjacent parenchyma attested to their rapid growth² (Fig. 1). They had a

² Some authors have remarked upon the fact that the induced or spontaneous adenomas of adult mice seem to be mostly situated at the pleural surface; but of the 15 adenomas found in animals 10 days old nine were well down in the parenchyma. It was noteworthy in this relation that the pulmonary tissue next the biggest tumors frequently showed compression on the side toward the pleura (Fig. 1), and occasionally there was an outward bulge over

TABLE 1
Adenomas in 10-Day-Old Mice from Urethanized Mothers
 (Only indubitable adenomas are included)

Urethane injections	Interval to birth	Mother No.	Adenomas	Time from first dose	Weight at death	Remarks
No.	days			days	grs.	
4	1	I	0	15	5	
		II	0	"	5	
		III	0	"	8	
6	1	IV	A-A A A 0	17	5.3 5 5 4.7	See Table 2 for litter mates
		V	A-A-A	"	4	" "
		VI	A A-A	"	7 6.6	" "
	2	VII	A 0	18	5.5 "	
	3	VIII	A 0 A-A A	19	5.5 " 5 4.5	
		CI	0 0	.	5.2 3.9	See Table 2 for litter mates
		CII	0		6.8	
		CIII	0 0		6 5.5	
		CIV	0		6	
		CV	0		5.6	
		CVI	0 0		6.5 6	
		CVII	0 0 0		8.3 8.2 "	
		CVIII	0 0		4.5 5	
		CIX	0 0		4.5 4.7	

Each A means an adenoma.

close general resemblance to the adenomas arising spontaneously in adult *C* animals and those induced in *A* mice with methylcholanthrene or urethane, and like them were either wholly glandular (Fig. 3) or partially compact (3, 11) (Fig. 2), their older central portion then being adenomatous. At some marginal spots the neoplastic cells formed a single layer on the walls of alveoli, as if extending along them with local heapings up.

Although the growths could easily be distinguished for what they were,—a recognition validated by study of the specimens from mice killed in later weeks,—they differed significantly in detail from the adenomas of adults. This matter will be considered further on.

To recognize the tumors in 3-day-old animals proved more difficult. Only three out of 10 of them had indubitable adenomas (Table 2), a single growth in each instance, but three others had what seemed to be early stages, wholly resembling those reported by Nettleship, Henshaw, and Meyer as appearing in urethanized adult mice (3), and comprehensively described by Grady and Stewart in their study of how adenomas come about after the intravenous injection of a colloidal suspension of methylcholanthrene (11).

The tumors were much less well developed than in the 10-day animals, as would follow from the brevity of the interval since the first urethane injection. The largest was found in the individual surviving for the longest time after the first injection of the mother (14 days), the next largest after the next longest time (11 days), while in the case of the least advanced growth only 10 days had elapsed. The largest (Figs. 4 and 5) lay deep in the lung, was markedly basophilic, and stood out sharply from the surrounding parenchyma. It consisted of the characteristic cuboidal cells, enclosing and partially filling a number of small spaces. A venule lay amidst it, as frequently happens in the forming adenomas of adults. It appeared multicentric where cut across, and its aspect suggested an origin from bronchiolar epithelium, but serial sections proved it to be a single growth lying well away from any bronchus or bronchiole. The lining of these latter stained a much paler blue.

Fig 6 shows the subpleural situation and discrete character of the growth found 11 days after the first exposure of the mother to urethane, and Fig. 7 reveals its characteristic morphology. Its cells are ranged in the usual single layer about small "acinar" spaces, and already it protrudes from the pleural surface. The parenchyma round about looks normal; as yet the cells lining the alveoli have undergone little flattening.

The 3-day-old animal which showed a growth only 10 days after the first giving of urethane had six litter mates and four of these were let live until 10 days after their birth, that is to say until 17 days after the first injection. Two had then a well developed adenoma each (Figs. 1 and 3), and a third had two of them (Table 1, offspring of mother IV). The growth in the 3-day-old animal consisted of a clump of cells, for the most part compact but covering part of an adjacent alveolar wall with a single layer (Fig. 10). They were sharply set off from their surroundings by their strong blue color and large, round, vesicular nuclei (Fig. 9). Mitoses were present. The clump had no direct connection with the epithelium of any bronchiole but lay well down in the parenchyma, and there was no cellular reaction round about it.

growths situated at some distance beneath it. The inference seems justified that a considerable proportion of the adenomas visible on the pleural surface of adult mice reach the surface secondarily as result of local pressure conditions as well as of enlargement. There is no need to invoke local differences in their initial distribution in the lung tissue.

The lungs of three other 3-day-old mice contained small discrete clumps of markedly basophilic cells with round, vesicular nuclei. They were solitary, and were situated in or on the wall of an alveolus. The instance pictured (Fig. 8) came from a sixth animal of the litter

TABLE 2
Adenomas in 3-Day-Old Mice from Urethanized Mothers

Urethane injections	Interval to birth	Mother No.	Adenomas	Time from first dose	Weight at death	Remarks
No.	days			days	gm.	
4	7	IX	A	14	2	
6	1	IV	0 A? A	10	2 1.8 1.9	See Table 1 for litter mates
		V	A?	"	2	" "
		VI	0 0 A?	"	2 1.9 1.9	" "
	2	X	A 0	11	1.9 1.9	
Controls		CI	0 0 0 0		1.7 1.6 1.6 1.5	See Table 1 for litter mates
		CX	0 0 A? 0		2 2 1.9 1.8	
		CXI	0 0		2.6 2.7	
		CXII	0		1.8	
		CXIII	A? 0		2 2.2	

A = characteristic adenoma. A? = dubious early stage.

of seven (Tables 1 and 2) dealt with in the last paragraph, the seventh proving negative. The clumps are queried in Table 2.

The Findings in Newborn Mice and in Embryos

From the relative size of the adenomas in 10-day- and 3-day-old individuals, it seemed likely that any encountered in still younger mice would consist of but

few cells. And in fact no indubitable adenomas have been come upon in 10 animals (from three litters) which were killed within 24 hours after birth, or in 14 embryos (from four litters) 18 to 20 days along. All the mothers had received six injections of urethane,—the last of them 1 to 3 days before parturition in the case of the young that came to term.

The lungs of mice less than 24 hours old have an alveolar lining that is but little flattened as yet, and often they are still partly atelectatic, a state of affairs rendering the recognition of adenomas almost impossible, as Tyzzer noted in his classical study of these growths in adults (12). The cells lining the alveoli of fetuses near term are basophilic and cuboidal, with round, vesicular nuclei, in other words closely resemble the cells of adenomas. It would manifestly be impossible to discern minute growths of this sort if situated in the parenchyma, but if they lay next the pleura or bulged it outward they might be perceived; and we have found what appears to be a forming growth (Figs. 11 and 12) in one of four fetuses removed from a female on the day after the last of six injections of urethane.

The mother animal had been selected by palpation as in mid-pregnancy, and hence the age of her fetuses was not precisely known; but the development of their lungs indicated that they were close to term, though they were only 22 mm. long as compared with 24 to 28 mm. for newborn mice from normal mothers. Mention has already been made of the frequent stunting effect of urethane on the embryos. Figs. 11 and 12 show that the problematic growth was discrete, bulging the pleura outwards, and that it consisted of cells with vesicular nuclei, ranged about an acinar (?) space. Nothing like it was found in the lungs of the other embryos, including three from the same litter, nor in those of any of the newborn mice.

The Findings in Sucklings of the A Strain

As already stated, adult mice of the A strain are far more liable to spontaneous adenomas than are those of the C breed (9), while furthermore the growths can be induced much more readily with methylcholanthrene (10). Hence we supposed that the offspring of urethanized A females might develop them earlier and in relatively great number. But on test an obstacle was met: the A animals injected during the latter half of pregnancy did very badly although much bigger than Cs at this time, and though only the standard amount of urethane (0.3 cc.) was given. Those receiving six injections either died or aborted or ate their young at birth, and so too with most of the mice which had four or five injections, the few that survived being so close to term at the time of the last as to give birth to their young on the day afterwards,—as did also the only animal that received three injections. It follows that the total interval between the first exposure of the young *in utero* to urethane and examination of them 3 days and 10 days after birth was, 7 to 9 days and 14 to 16 days respectively, that is to say was shorter than in the case of C mice. Furthermore the embryos had been exposed to much less urethane, both because of the fewer injections and because of the large bulk of the mother. Whether for these

reasons or no, the growths have been infrequent and tiny in individuals 10 days old (Figs. 13-15), actually comparable with those in 3-day-old C mice. For this reason no extensive study has been made of them. None was found in several A animals examined when 3 days old.

The Early Stages of Adenoma Formation

As already remarked, the early stages of the growths resembled those observed in adult mice given urethane or methylcholanthrene (3, 11).

First seen in the parenchyma were occasional discrete clumps of cuboidal or rounded cells situated in or on the walls of alveoli (Fig. 8), and with cytoplasm that stained an intense blue, sometimes very dark, contrasting so sharply with the hue of the normal parenchyma as to attract attention at low magnification. These cells had round or slightly oval vesicular nuclei, unlike those of ordinary alveolar elements, which are mostly dense, almost pyknotic, and more or less oblong. The clumps seemed punctate in origin and almost devoid of capillaries, unlike the richly vascularized alveolar tissue about them; yet their components looked very active. The growths of Figs. 9, 10, and 15 represent the earliest stage at which an adenoma gave definite signs of what it was. Here the cells have heaped up into a compact mass, but in Fig. 10 an adjacent alveolus is partly lined with them; mitotic figures can be seen. The first growth of Figs. 6 and 7 looks as if it had been adenomatous from the first. The tumor of Figs. 4 and 5 is expressive of further development. None of the growths was multicentric.

In late fetuses and sucklings up to 10 days old, of both the C and A breeds, spherical or discoid aggregates of cells were frequently found just beneath the pleura, which were at first taken for compact adenomas, and with the more reason because of the frequency with which the growths are found in this situation. They occurred irrespective of whether the mother had received urethane, and consisted of more or less cuboidal elements with round vesicular nuclei and cytoplasm staining blue,—cells not to be distinguished individually from those of adenomas. Sometimes the aggregates were spherical, lying tangential to the pleura amidst the lung substance, but more often they were discoid and protruded on its surface. Mitotic figures were frequent in them, far more so than in the lung parenchyma round about; and often the cells partially filled adjoining alveolar spaces. But it was noted that some of the masses, though appearing spherical in cross-section, were really fusiform, running through many sections, and furthermore that a thin membrane formed by reduplication of the pleura was attached to the surface either over them or near by; and the fact became evident that the masses were mere "mooring clumps" for membranes joining one lobe of the lung loosely to another. Fig. 16 shows a characteristic example. Frequently the clumps were situated at the sharp edge of a lobe or next it, that is to say at sites where adenomas are prone to occur; but now and then one was on the shoulder of a lobe or its rounded convexity. Occasionally a small lenticular mass of blue-staining cells like those composing a mooring clump was to be seen closed in between the layers of the membrane, well away from the lung surface. Even where the cells of a clump filled alveolar spaces, they were never ranged in the glandular pattern of mature adenomas; yet the fact remains that if no membrane had been attached next them they could not have been distinguished with certainty from compact tumors of such kind. For this reason we have accepted no subpleural growth as an adenoma unless it had typical features, and hence undoubtedly some have been omitted from Tables 1 and 2. The pleural nodule of Figs. 11 and 12 lay on the convexity of a lobe, had no membrane inserted over it, and an acinus seemed to have formed within it; yet because of the existence of "mooring clumps" its status is problematic. These are not infrequent in embryos.

Solitary, almost spherical giant cells 30 to 40 μ across, containing two or three vesicular nuclei, were present now and again on the alveolar walls of 3-day and 10-day animals, never more than two or three of them to a lung. None was found where adenomas were forming, and they seemed to have no relation to this process. Quite often in both C and A sucklings,—from control as well as urethanized mothers,—there were what may be called “polymorphonuclear balls,”—small, sharply demarcated, spherical aggregates of cells staining blue like adenoma cells, but with nuclei of horseshoe shape or resembling those of the blood polymorphonuclears, though coarser and less pyknotic. The cells had no granules, showed no division figures, and the balls formed of them appeared in excellent state, without sign of central necrosis. Usually they lay just outside some capillary in the alveolar wall, which appeared patent and normal. Wholly different in aspect from adenomas, their significance is not clear and their ultimate fate has not been traced. None has been come upon in embryos.

The Findings in Control Animals

It is singular that the literature contains no observations on how soon spontaneous adenomas begin to form. The scarcity of mitoses when they first come to attention in adult mice makes plain that their recent growth has been exceedingly slow, and it may well be that they originate early in life. Hence our diligent search for them in the control young of the present work,—the more careful because the tumors would doubtless be small, and perhaps solitary.

The lungs of one of 13 normal C mice 3 days old, examined *in toto* section by section, had a discrete clump of dark blue cells, in the parenchyma far from any bronchiole (Fig. 22); but both the cells and their nuclei were smaller and denser than those of the adenomas found at this time in the offspring of urethanized females. In another control mouse 3 days old several adjacent alveoli were almost filled with clumps of blue cells. Their nuclei were smaller than ordinary and oblong in some instances, yet the resemblance to a forming adenoma was great and at one spot the cells were ranked in two parallel rows (Fig. 23), as often happens in such growths. In the lungs of three out of 16 normal animals 10 days old small compact clumps have been found of basophilic cells wholly resembling the one pictured in Fig. 8 as coming from a 3-day-old mouse of an urethanized mother. There was only one such clump in each animal.

In sum, the findings give some support to the possibility that adenoma cells may have been present in the pulmonary tissue of the young normal mice. Their existence could not have been excluded in any case, since it would be impossible to identify them if scattered singly. This much is certain however, that the lungs of the controls contained no adenomas identifiable as such.

Origin of the Adenomas

Some workers are convinced that the adenomas called forth by urethane in adult mice arise on the basis of chronic inflammation of the pulmonary tissue, due either to the substance itself or to intercurrent infection. No sign of any such course of events has been observed in the young animals of the present work. Orr believes that the preliminary inflammation may have disappeared by the time the adenoma is well formed (13).

Urethane is known to produce ascites and to cause injury to several organs, largely in consequence of capillary damage. Orr reported that it set up chronic pulmonary inflammation in the outbred stock mice employed in his experiments, and Winchester and Higgins (14) found that it induced more or less pulmonary edema in animals of the C strain. The quantity of urethane injected into the pregnant females of the present work frequently exceeded the tolerable maximum, many of them dying; and often the fetuses and young of the surviving animals were abnormally small, as already mentioned, while occasionally the development of the lungs seemed retarded; yet nowhere were any local cellular anomalies other than adenomatous change perceptible in the pulmonary tissue. Furthermore there was no cellular reaction about the forming growths, as the figures sufficiently demonstrate.

The lungs providing material for the conclusion that adenomas are secondary to inflammation have generally been the subject of consolidating infections. The C strain is no exception in this latter respect. Mature animals of our colony often develop a consolidation which slowly involves most of the pulmonary tissue and eventually proves fatal. The disease is obviously infectious, spreading rapidly amongst cage-mates; but its cause is still undetermined. The lesions it produces have no resemblance to adenomas. The bronchioles seem to be first affected, cellular exudate accumulating within some of them; more and more are implicated; large and small mononuclear cells accumulate round about them and about the blood vessels; and the alveoli become filled with swollen, desquamated elements. The gross result is consolidation. In all these respects the findings are like those in the "grey virus disease" described by Andrewes (15). Some of the urethanized mother animals, killed with their young 60 or 70 days after parturition, had developed the malady and hence were discarded together with their offspring, but the lungs of the great majority appeared normal, save for adenomas, and those of the embryos examined and of the 1-day- and 3-day-old sucklings gave no microscopic evidence of the disease. In a single 10-day-old animal its beginnings were found.

Manifestly the best method to determine whether the consolidating disease prepared the way for the adenomas was to try to induce the latter in the young of a breed with healthy lungs. This was one reason for the tests of A mice. Our colony of them is remarkably free from pulmonary inflammations and the "lung disease" in special has never been found. Nevertheless adenomas arose in the young of females urethanized while pregnant. There was no cellular reaction about the growths (Figs. 13-15).

These facts, considered with what has already been said, prove that the adenomas induced in young mice were not secondary to pulmonary inflammation but primary in origin. Nettleship, Henshaw, and Larsen decided that the growths they induced with urethane in adult animals ordinarily arose from tissue which had undergone "little or no injury," and Grady and Stewart found no trace of preliminary pulmonary disorder in connection with the early stages of the adenomas due to methylcholanthrene.

Derivation of the Growths

Opinions have differed widely on whether pulmonary adenomas originate from alveolar cells or from the epithelium of the bronchial tree. None of the growths in sucklings had any direct connection with this latter; they were everywhere surrounded by parenchyma. All were arising, or appeared to have arisen, either from the alveolar wall or from elements of alveolar character lying

next the pleura, and the resemblance to bronchial epithelium was but slight. In the mother animals on the other hand, examined 70 days after parturition but of indeterminate age, and doubtless having in some instances "spontaneous" adenomas antedating the urethane injections, growths were not infrequently encountered which were composed of elements like those lining the small bronchioles (Fig. 17) and staining nearly as pink. The morphological resemblance was complete, except that the tumor cells were seldom markedly cylindrical, a difference which might have been consequent on pressure factors. In both instances a large proportion of the nuclei were round or slightly oval, and vesicular, as in the growths of sucklings, but many others were oval or oblong and almost pyknotic, often notably big and sometimes then with much more cytoplasm than usual about them. When oblong they were frequently ranked with their long axes parallel. All gradations between the two types were present. The resemblance to bronchiolar epithelium can be seen in Fig. 18. The growth providing this figure, made for ease of comparison, was exceptional in protruding into the lumen of the adjacent bronchiole; but other adenomas with identical features (Fig. 19) were wholly isolated amidst the lung parenchyma, as serial sections proved.

Amount of Exposure to Urethane as Determining the Incidence of Tumors

In Table 3 are listed all those cases in which the urethanized females were examined, as well as their remaining offspring, 60 to 70 days after parturition. The age of the females was not known, but they were sturdy multipara, several months old at the least, and some must have been well on the way toward having "spontaneous" adenomas, as has just been remarked. They, not their embryos, bore the immediate brunt of the urethane. On both these grounds one might have supposed that they would have had adenomas more often than their young, when all were eventually killed. And this was so, 10 of 14 mothers showing them in the gross on the pleural surface as compared with 10 of 49 young. Yet they were few at most in the mothers and usually solitary. Repetition of the injections had but a slight effect; in only two of five mother animals which had received four or six injections were there definitely more tumors than in those which had received but one.

Larsen noted that the number of adenomas present after 6 months in the offspring of A mice receiving urethane while pregnant largely depended upon when the substance had been given (6). There were at least five times as many when it had been injected during the final 24 hours of gestation as when it had been administered earlier, and furthermore every animal had them, instead of a large proportion. The reason, Larsen concluded, was that more blood carrying urethane reached the fetal lungs in the final hours before birth. No such differences with time of injection have been perceptible in the present experiments. The microscopic findings in 10-day-old animals carry some weight in

TABLE 3

Peripheral Adenomas in Mothers Urethanized While Pregnant and in Their Offspring
(Findings 60 to 70 days after parturition)

Urethane injections	Interval to birth	Mother	Offspring		Adenomas		Remarks
			Age	Mouse	Presence	Size	
No.	days		days			mm.	
1	2	A			0		
		•	70	a	+	1	
				b	0		
				c	+	1	
				d	+	1	
				e	+	1-1	
	6	B			0		
			70	a	+	2	
				b	+	1-1	
				c,d	0		
		C			+	1	
2	1		67	a,b,c,d,e,f	0		
		D			+	1	
			67	a	+	0.5	
				b,c,d,e	0		
3	1	E			0		
			70	a	0		
		F			0		
			70	a,b,c,d,e,f	0		
4	1	G			+	1	
			70	a	+	1.5	
				b,c	0		
		H			+	1-1	
5	1		70	a	0		
		I			+	0.5	
			70	a,b,c,d,e	0		
		J			+	0.3	
6	2		60	a	+	0.3	
		K			+	1-1-1-1-1	
			70	a,b,c,d	0		
		L			+	1	
7	4		70	a	+	1	
				b	0		
		M			+	1-1	
			70	a	+	2	
8	2			b,c,d,e	0		
		N			+	1-1-1-1-1-1	

Cardioma in other lung

this relation because their entire lungs were examined microscopically. It will be seen (Table 1) that the incidence of adenomas in the litters from mothers getting six injections of urethane was only dubiously larger when the last had been given within 24 hours of parturition than when it had been given 2 or 3 days previously. The findings in the young killed 60 to 70 days after birth stress the same point. The offspring of females receiving four injections, the last within 24 hours of parturition, had no more tumors visible in the gross than those from mothers receiving the final injection 4 days previously (Table 3).

Nor did repetition of the injections into the mothers make adenomas any more frequent in the young. Actually the incidence of these growths in the offspring of the four mothers receiving urethane only once was considerably greater than it was in the young born to the eight animals getting it three to six times, seven out of 20 young mice in the first category having tumors visible in the gross as compared with four out of 22 in the second.

Mention has been made of the fact that a large proportion of the pregnant mice receiving urethane several times did badly and gave birth to young that were far under weight. Here was a possible reason for the differences just brought out. For Tannenbaum has shown that the appearance of spontaneous adenomas in adult animals can be checked or even prevented by underfeeding them (8); and the offspring of mothers getting urethane three to six times might have been more poorly nourished than those from females receiving it only once. But all the young of Table 3 appeared healthy when they were killed 60 to 70 days after birth, and while some were unusually small the two groups did not differ significantly. Of course early differences crucial to the development of adenomas might have been ironed out by this time, but the findings in the 10-day-old mice of Table 1 speak against the influence of any such differences. It will be seen from the weights there given that the individual with most growths (offspring of mother V) was the smallest of the seven killed 17 days after a single injection of urethane into the mother,—weighing 4 gm. as compared with a maximum of 7 gm. An animal having three peripheral adenomas, each a millimeter across when it was killed 72 days after birth from a female repeatedly injected with urethane weighed only 13 gm. at this time instead of the normal 20 to 24 gm., and it had weighed only 2.8 gm. when 10 days old and been almost devoid of hair then. It is not listed in Table 3 because its mother was not examined at the same time.

A better reason why repetition of the injections failed to result in more growths is perhaps to be had in the difficulty of inducing adenomas in adult animals of the C strain. No previous observations with urethane are on record, but methylcholanthrene gives rise to the growths very slowly in C mice, as already mentioned, and the number is small for a long time. Only toward the 20th week after intravenous injection of the hydrocarbon did they appear

in Shimkin's test, whereas in A mice there were more than 24 to an animal by the 6th week (10). They were never many in our C animals, and hence chance must have entered largely into the gross findings of Table 3. Yet the results cannot be wholly accounted for on the basis of these facts. The C embryos exposed to urethane in the mothers receiving but a single injection of it proved so responsive that adenomas followed in a large proportion of instances (Table 3), the growths arising very rapidly (Tables 1 and 2). Multiple injections failed to elicit them in any greater number. One injection into two pregnant females 2 days before parturition resulted in adenomas in six of their nine offspring, whereas three and four injections into five mothers, the last given within the final 24 hours of gestation, yielded growths in only two of 14 young (Table 3). These data, which go against all experience with adult mice, strongly suggest the existence of litter differences in the potentialities for adenoma formation. No sex differences were observed.

One might have supposed that the young of the two females receiving most urethane and themselves developing multiple adenomas in consequence (mothers K and N, Table 3) would have had growths most frequently. Actually their young had none,—at least none visible in the gross on the pleural surface. But the pleural index must sometimes be misleading when pulmonary tumors are few. Nevertheless Table 3 in its entirety seems to indicate that the offspring of individual C mothers developing adenomas in the gross after urethane injection possessed no greater tendency to such growths than the young of females in which none appeared. Using the pleural index Lynch was unable to obtain any evidence of a maternal influence on the incidence of pulmonary adenomas in tarred mice (16), and Bittner and Little found none in the case of the spontaneous growths (17).

Age as Affecting the Adenomas

Nearly everyone studying the adenomas of adult mice microscopically, whether spontaneous or induced, has remarked upon the scarcity of mitoses. Yet in the growths of the 10-day sucklings they were so abundant that often nearly every section showed them (Fig. 1), five being visible in a single high-power field of the microscope in one instance. There were other signs too of pronounced cellular activity. The adenomatous pattern was often ill-defined, and the individual cells were less differentiated and more basophilic than in the growths of adults (Figs. 1 and 2). The nuclei were almost uniform in size, round and vesicular, with little chromatin and this mostly margined; the cytoplasm was scant.

The tumor animals had other animals killed 60 to 70 days after parturition were far more ~~various~~ ^{various}. All were frankly adenomatous in arrangement and mitoses were few or wholly lacking (Table 4); many of the growths stained purple and now and then the color was pink. Occasionally one consisted

entirely of cells with vesicular nuclei like those of 10-day-old mice, but in most instances a considerable proportion of the nuclei stained a dark, even, almost pyknotic blue (Figs. 17-19), and often then they tended to be relatively large, and oval or oblong instead of round. Not infrequently when this was the case they had an unusual amount of cytoplasm about them (Fig. 17). Some of the cells with these features may have been intrusive, but the majority were ranged side by side with the ordinary elements having round, vesicular nuclei, and there were all gradations between the two. The differentiation of the neoplastic elements,—for such it obviously was,—culminated in cells which resembled those lining the bronchioles, though not quite so acidophilic (Fig. 17). Often they were in rows like these.

In sum, it was plain that the adenomas of 10-day-old mice consisted of actively multiplying cells, which for that reason, at least in part, were undergoing but little differentiation, whereas those of the mother animals were indolent and had largely differentiated.

A corroboratory indication of these contrasting states was found in the relative size of the growths encountered in mothers and young respectively after 60 to 70 days. Although the mothers were by then well along toward the age when some might have had spontaneous adenomas visible in the gross, and although they had received urethane directly, the largest of their tumors were only half as big as those in certain of the young (offspring of mothers B and M, Table 3). Indeed the average adenoma in these latter was as big as any in the mothers. Active proliferation must have kept on during some weeks for them to have reached such a size.

In order to learn whether cell multiplication was still continuing in any important degree comparative counts were made of the mitotic figures in some of the growths of mothers and young. The undertaking was not comprehensive; just enough was done to answer the question posed. The findings are given in Table 4.

It will be seen that no mitoses were present in the two adenomas, each a millimeter across, of mother M of Table 3, nor any in a smaller growth disclosed on serial section of the lungs. There were a few in the 2 mm. adenoma of one of the young from this mother, as also in another growth of considerable size which lay deep in its lung parenchyma. But evidently cell division had almost ceased, and this was the finding also in the notably big adenomas of other young animals, the progeny of mothers A and G. Some of the growths in these young showed no mitoses whatever, and their maximum frequency was no greater than in several of the tumors of mother K. It may be seen, then, that this animal was one of the two which had multiple adenomas visible in the gross, after the repeated injection of urethane, and that none of her very young showed any tumors (Table 3).

The conclusion seems warranted that proliferative activity in the adenomas

TABLE 4

Mitoses in the Adenomas of C Mice Urethanized While Pregnant and of Their Young
(Findings in growths present 60 to 70 days after parturition)

Days	Urethane injections	Mother	Young	Size of tumor	Cells examined	Mitoses		Remarks
						Actual No.	No. per thousand cells	
70	1	A		no.	3,000	0	0	Whole tumor searched
			a	1	24,000	0	0	
			c	2	42,000	2	0.05	
			"	1	40,000	1	0.03	
			"		18,500	1	0.05	Whole tumor searched
			"		10,400	0	0	Tumor partly lost
	3	G			9,400	1	0.1	Tumor partly lost
			a	1.5	44,500	0	0	
60	4	J		0.3				
			a	0.3	24,500	5	0.2	Growth in mother lost
70	4	K (Her four young were negative in the gross.)			22,600	3	0.13	Whole tumor searched
				1	33,400	4	0.12	Whole tumor searched
				1	20,500	2	0.1	Tumor partly lost
				1	19,200	3	0.16	Whole tumor searched
				1	18,800	0	0	" " "
				1	15,700	3	0.19	" " "
					14,000	4	0.29	" " "
					8,400	2	0.24	" " "
					4,350	3	0.7	" " "
		M		1	63,000	0	0	Whole tumor searched
				1	33,400	0	0	" " "
					12,100	0	0	
			a	2	598,400	19	0.03	Whole tumor searched
			"		61,200	2	0.03	" " "

The growths for which no size is recorded were either microscopic or had lain hidden in the parenchyma. All recognizable stages of mitosis were counted.

of the young animals had almost ceased by the time they were 60 to 70 days old, being by then nearly on a par with that in the mother animals. And there was substantial evidence to this effect in the morphology of the growths, which now exhibited not infrequently a differentiation as complete as that in

the tumors of adults. It was marked in some of the biggest adenomas of the young (Figs. 20 and 21). Growths in the same individual often differed widely in such respect, just as happens in adults.

DISCUSSION

The main aim of the present work, like that of other experiments previously reported (1), has been to learn whether the cells of embryos possess the ability to undergo neoplastic change. On first inspection the facts seem to leave no doubt of this for the lung, yet they cannot forthwith be accepted as proof.

First one must know whether the growths appearing in the offspring of urethanized mothers are true pulmonary adenomas, and if so, whether the latter are really tumors. On both points the answer is yes. The growths have been followed through every stage to the form found in adults, and they exhibit the familiar, distinctive characters of pulmonary adenomas. All workers with these latter are now agreed that they are genuine neoplasms. Both the spontaneous and the induced growths have been transplanted successfully (18), and while some have become carcinomatous on passage others have retained their initial traits. Adenomas are the first and most frequent tumors to arise from pieces of mouse embryo lung implanted with methylcholanthrene in the leg muscles of adults of the C strain (4), and several obtained in this way have been transplanted. One has now been propagated in five successive groups of adult hosts, and it has retained its original adenomatous morphology although in the course of months it forms huge tumors, killing the hosts. So convincing is the evidence that the adenomas are genuine neoplasms that we have deemed it unnecessary to try to transplant those obtained in sucklings. Doubtless for the same reason Larsen has reported no transplantations of the adenomas present in 6-months-old A mice from urethanized mothers (6).

So rapidly is urethane excreted that its direct effect can only have been exerted upon the embryos *in utero*, organisms that is to say which are protected from many extraneous influences, notably most of the infections. This state of affairs has provided opportunity to gain light on several moot points.

Much uncertainty has existed as to whether the adenomas arise from alveolar cells or from bronchial epithelium. Tyzzer, who first studied them comprehensively, concluded that they could be of either origin (12). Those we found in sucklings were wholly surrounded by alveolar tissue, and the evidence of early stages was all to the effect that they had originated from alveolar elements, like those induced in adults by methylcholanthrene (11). None had any direct connection with a bronchiole. But needless to say, as an adenoma grows large it must often fill the space into which a bronchiole opens and may even project into its lumen. The cells of the tumor of Fig. 18, in which this was the case, were not joined to those lining the bronchiole, yet a union might on occasion take place secondarily since experiment has shown that elements as widely

different as those of regenerating epidermis and of a carcinoma of prostatic origin can join in a layer (19). Furthermore the cells of pulmonary adenomas in adults sometimes resemble the epithelial elements lining the bronchioles (Fig. 17). It seems likely that these phenomena have provided the grounds for the belief that the growths often take origin from the bronchiolar lining,—the more so as the evidence on this point has been mostly obtained through studies of large, well established growths under conditions complicated by bacterial infection, atelectasis, and consolidation. If adenomas do arise now and again from the epithelium of the bronchial tree the occurrence must be highly exceptional.

The derivation of the cells lining the alveolar wall has long been the subject of controversy, some investigators holding that they are epidermal in nature and others that they are mesodermal (20). The changes taking place secondarily in the adenomas developing in the young of urethanized mothers provide evidence in this matter. Though the growths originated from the alveolar wall and had no direct connection with the bronchiolar epithelium, their cells not infrequently took on a resemblance to the latter within 60 to 70 days (Fig. 20). Occasionally in the growths of the mothers the resemblance was absolute (Fig. 17). Recently one of us (21) has found that the cells of the alveoli formed after the transplantation of fragments of mouse embryo lung to adult hosts may undergo metaplasia to stratified squamous epithelium if exposed to methylcholanthrene. The change may occur at spots distant from bronchiolar epithelium, as Passey has noted in rat lungs chronically inflamed by bacterial infection (22). These facts, taken together, make plain that the cells of the alveolar wall are pluripotential despite their specialization, and that they are epithelial in character. The hypothesis might be put forward as alternative that bronchiolar elements lie scattered amongst the alveolar ones, all undiscerned, and that the adenoma cells and squamous epithelium derive from these. But if this is the case why do not adenomas arise often from the bronchiolar lining instead of rarely if at all?

Certain workers with the spontaneous adenomas of adult mice and those induced with urethane believe that both arise on the basis of inflammatory lesions. If this were the fact the tumors occurring in the young of urethanized females could have resulted from neoplastic changes taking place after birth, in animals living long enough; the 6 months' existence of Larsen's mice would have provided abundant opportunity for this sequence of events. The present findings exclude it. Neither in the embryos removed from females receiving urethane repeatedly nor in their recently born offspring did any pulmonary inflammation occur which might have provided a basis for later adenomatous change; and no reactive proliferation or accumulation of lymphocytes or macrophages took place about the growths in the young animals (Figs. 1-14). The conclusion seems warranted that the neoplastic changes which found expression

in the adenomas of sucklings were not secondary to inflammation but primary in origin.

The action of urethane to elicit tumors has excited much comment, for it has appeared peculiar in several respects. The simplicity of the substance as compared with most oncogens has seemed to set it apart,—though subcutaneous injections of sugar cause sarcomas to arise in the mouse, and hydrochloric acid thus introduced will do so in rats. The finding has also seemed remarkable that urethane induces no growths except pulmonary adenomas,—though ergot gives rise to neurofibromas only, and Scharlach R to hepatomas, examples which need no longer be cited, now that urethane has been shown to elicit hepatomas as well as adenomas (23). The fact that a single exposure to the substance suffices for neoplastic change has also aroused speculation,—although a single painting of mouse skin with methylcholanthrene results in cancer in some strains of animals. More extraordinary is the finding that pulmonary adenomas arise in the absence of any visible tissue damage. Grady and Stewart (11), noting this of the adenomas induced with methylcholanthrene, were led to ask whether the chronic “precancerous” tissue alterations, which so generally precede the occurrence of tumors as almost to enter into any definition of them, are really essential to their origin. The question is worth asking again.

There remains to consider, of the peculiarities of urethane, the rapidity with which it induces neoplastic change as indicated by the present work. Here a distinction must be made between the preliminaries to such change and its actual occurrence. Though the preliminaries frequently extend over a long time the change itself takes place rapidly, according to all observation with oncogenic agents; it is as if a trigger had been pulled. The generality of agents press gradually upon the trigger; only its eventual click is abrupt. Urethane would seem to pull it hard. But another possibility exists, that the substance merely stimulates the proliferation of cells already neoplastic. Several authors have thought that this is its mode of action. The occurrence of problematic cell clumps in our young control mice might be viewed as supporting such a conception, and the extraordinary rapidity with which adenomas arose in the animals urethanized *in utero* accords well with it. Observations on the point are under way.

According to an axiom now well authenticated through experiment, the greater the natural liability of mice to this tumor or that the more readily can the growth be elicited by the application of an oncogen. Everything that is known of the response of various breeds to urethane falls in with this generalization. Nettleship, Henshaw, and Meyer (3), who first demonstrated that the substance induces pulmonary adenomas, noted also that it elicited many more of the tumors in A mice, the breed most prone to them spontaneously, than it did in C3H animals. Cowen (24) obtained data in R III, CBA, and C57 mice

which accord with this finding as do also the present results with adults of the C strain. Henshaw and Meyer (7) believed that the adenomas began to form within 1 to 2 months after the injection of urethane into 6 to 8-weeks-old animals of the A strain.

From all this one might have inferred that adenomas could scarcely have developed in animals of the C strain only a few days old. But the conditions of test were exceptional. Urethane is so diffusible that it must have reached the embryos in quantity. It was given in the maximum amount tolerable and the exposure of the pulmonary tissue to it was intensive; mice have not previously been injected with it day after day. Furthermore the tumors arose under conditions making for their rapid enlargement; embryos and newborn animals are notably favorable hosts for implanted neoplasms (25), the growing organism providing in abundance the stroma and vascularization that tumors need. And there was a deeper reason why the adenomas appeared so soon and contained mitoses in profusion, namely the state of the cells engaged in producing them. They were already vigorously proliferating to form the lung when they came under the influence of urethane. That they were behaving in this way because of an innate urge has been decisively proven by Cohn and Murray for the chick embryo (26) and is sufficiently attested for the mouse by the continuing growth of fragments of embryo lung after implantation in adult hosts. The adenomatous change superimposed upon the cells such further activity as enabled them to multiply even more rapidly than their normal fellows,—an advantage which became increasingly manifest when the proliferative activity of the latter fell off after birth.

These findings bear on what is implicit in the neoplastic state. Widely various examples are on record of the extraneous stimulation of tumor cells; numerous substances have been found to promote the growth of tumors produced experimentally in animals and one often sees the phenomenon clinically, e.g., after a growth becomes infected with pus-producing organisms, or when testosterone urges on the prostatic carcinomas of man. All such happenings are comprehensible because the agents urging the growths on stimulate normal tissues as well; they act as adjuvant influences merely, and when they are no longer present their influence lapses. The happenings after adenomatous change has taken place in the young of urethanized females stand in a different category. In their case stimulation resulting from neoplastic change is superimposed upon a proliferative activity natural to the cells of the very young organism. Yet again the relationship is not enduring. As the animal becomes mature and its normal pulmonary cells almost stop multiplying, the tumor cells cease to nearly the same extent. Now their only advantage is that which the neoplastic state itself brought with it, and under the circumstances obtaining in the adult animal this suffices for but the slowest proliferation. The tumor cells have remained susceptible to the normal ageing influences. No longer

active, they differentiate until they more or less closely resemble the normal bronchiolar epithelium.

These facts appear to provide a partial answer to the question whether the period in the life of the organism at which cells become tumor cells has any importance for the neoplastic process set up within them. The period has indeed an influence in the case of the growths under discussion. When adenomatous change takes place in the very young organism it increases the activity of cells already possessed of a natural tendency to divide; and for this reason, as well as because of their highly favorable environment, they proliferate more rapidly than the adenoma cells of adults. But the relationship which has this consequence is no more than additive; and the most effective of its factors, those due to youth, soon wane and are gone.

Although the urethane acted upon embryos and the resulting adenomas were perceptible within the first days after birth, this does not mean necessarily that the pulmonary cells became tumor cells *in utero*. The relative size of the growths in the 10-day- and 3-day-old animals, and the failure to find any with certainty in fetuses and animals just born prove that their formation took place almost wholly after birth. But it could scarcely have been otherwise. The longest interval from the first urethane injection to parturition was 11 days (Table 2), and during the first 2 of these the lungs had barely begun to form. Nevertheless when the animal was killed, 3 days after birth, or after 14 days in all, an adenoma of considerable size had developed (Figs. 4 and 5). In no other instance of the sort was the elapsed time so long. The interval before birth in a second 3-day animal with a smaller tumor (Figs. 6 and 7) was 8 days, the total elapsed time 11 days; and in a third mouse, with a still less developed growth (Figs. 9 and 10), 7 days and 10 days respectively. The length of postnatal life was constant in these instances, only the prenatal interval varied. Yet much cannot be made of this circumstance as bearing on the size of the tumors, since the urethane may not have brought about adenomatous change until it had been given several times.

While the evidence is strong that neoplastic conversion was consummated *in utero* the possibility still must be considered that the urethane merely rendered the young animal susceptible to the influence of some extraneous tumor-producing agent reaching it immediately after birth. One thinks of an agent like that responsible for mammary cancer in the mouse, which is passed on from mother to young in the milk. But the data of Table 3 provide no support to this conception, disclosing as they do no correlation whatever between the incidence of adenomas in mothers and their young. The results of four or six injections of urethane are especially noteworthy in this regard; though the mother animals in some of these instances developed multiple adenomas, their young showed almost none. This is the more significant in view of the cumulative effect of urethane to elicit adenomas in adult animals, the rapidity with which these de-

clared themselves in the gross in young animals; and the fact that the offspring of females receiving urethane only once often showed them (Table 3). It would appear that, for reasons unknown, potentialities for the growths were slight or lacking in the embryos of those mice that happened to be many times injected. The existence of small islands of cells resembling the first stages of adenomas, in some of the control baby mice but not in others, may have a bearing on why the growths appeared in only a proportion of the individuals exposed to urethane instead of in all of them.

As already stated, the observations here recorded were made, like others from this laboratory, to learn how soon in the life of the organism cells possess the ability to become tumor cells. Growths arise so rapidly and in such diversity from mouse embryo tissues, after transplantation with methylcholanthrene to adult mice, as to make it well nigh impossible to suppose that extraneous actuators resembling those now known (the tumor-producing viruses), and entering after birth, are responsible for the generality of neoplastic changes. The facts all have indicated that the cells of the embryo possess the ability to become tumor cells (1). The present demonstration that the injection of urethane into pregnant mice causes adenomatous changes to come about so quickly in the young they carry that tumors are perceptible almost at once after birth makes it difficult to avoid the conclusion that the pulmonary cells of mice in mid-fetal development are capable of being neoplastic. This capability would appear to exist earlier in the life of the organism than the one for mammary tumors, which is conferred at the first nursing. Evidently each type of neoplasm must be studied for itself in such relation.

The relative share of the intrinsic and the environmental in neoplastic change is amongst the most deep-going questions in cancer research. Yet only the environmental factors have been inquired into with particularity thus far for the good reason that practically all observations have been made of necessity upon growths coming to attention after birth,—upon those developing in organisms which had passed out from the protection of the uterus into a world in which they were beset by oncogenic agents. Even the hereditary and familial tendencies to tumors, manifest in inbred strains of mice, have been perceptible only in terms of postnatal happenings. Gideon Wells could find but 66 valid instances of tumors in newborn infants, none of them carcinomas (27),—a negligible number, it may be remarked, in comparison with the instances of disease due to viruses reaching human embryos *in utero*. True, hydatidiform moles and chorio-epitheliomas are due to neoplastic changes in a tissue of embryonic origin: but the chorionic cells are not shielded by the natural contrivances whereby the embryo itself is protected; on the contrary they are even more exposed to environmental factors than the majority of the cells of the mother, bathed as they are directly in her blood. The cells composing embryonic rests and the frank embryomas and teratomas from which tumors

take off in later life are not only subject to the same environmental influences as the cells of the host but are exposed to special hazards, often becoming the seat of pathological disturbances which may render them exceptionally liable to neoplastic change.

SUMMARY AND CONCLUSIONS

The observation that adenomas develop very rapidly in the pulmonary tissue of mouse embryos implanted together with methylcholanthrene, in adult animals, has led to tests of the neoplastic potentialities of this tissue *in utero*. C strain females in the latter half of pregnancy were injected with urethane and the lungs of their young were searched for adenomas. None could be perceived with certainty in embryos at term or in mice just born, but they were several times found 3 days after birth and they were frequent and much larger in 10-day-old animals. The controls showed none. After 60 to 70 days they were often visible in the gross. Corroboratory findings were obtained in A mice. No parallelism could be perceived in the incidence of the tumors in mothers and offspring.

The adenomas arose from tissue devoid of any sign of preliminary local disturbance. Mitoses were abundant in them and they grew rapidly for a while, but within 2 months cell division had almost ceased. By this time however many of the neoplasms were as big as any adenomas in the urethanized mother animals and in some instances twice as big. While growing fast they underwent little differentiation, but this took place when proliferation slowed and in consequence the tumors came to have the morphology of the spontaneous and induced adenomas of adults.

The neoplastic cells were derived from alveolar elements, yet in proportion as differentiation of them occurred they came to resemble the epithelial cells lining the small bronchioles. Occasionally the resemblance to bronchial epithelium was complete, save that the cytoplasm of the tumor cells was slightly basophilic.

The following conclusions seem justified:—

1. The injection of urethane into pregnant female mice of the C strain frequently initiates the development forthwith of pulmonary adenomas in the young she is carrying.
2. Some of the pulmonary cells of mouse embryos well along toward term possess the ability to be neoplastic.
3. The adenomatous change finds swift expression in young creatures because of conditions implicit in their youth. The rapid proliferation of the tumor cells is almost entirely due to these conditions, not to the neoplastic state as such.
4. Adenomatous change prior to birth is intrinsically the same process as that occurring in the adult creature.

5. The adenomatous state does not prevent the cells of young mice from undergoing the maturation that takes place in normal elements of the same sort as the organism grows older. Though the proliferative activity natural to youth and the unnatural activity consequent on neoplastic change are summated in the young organism, they still are separable.

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EXPLANATION OF PLATES

The photographs were made by Mr. J. A. Carlile.

All of the sections were stained with eosin and methylene blue.

PLATE 24

FIG. 1. Adenoma in a 10-day-old mouse from a mother that had received six injections of urethane, the last within 24 hours of parturition (mother IV of Table 1). The growth lies near the lung surface and has compressed the parenchyma between it and the pleura,—seen on the left. Its cells are more basophilic than those of the parenchyma about them and they nearly all have large, round vesicular nuclei. The arrows point to two of the many mitotic figures. $\times 505$.

For other growths in animals of the same litter see Figs. 3, 9, and 10.

FIG. 2. Adenoma in a 10-day-old animal from another mother getting six injections, the last of them 3 days before parturition (mother VIII of Table 1). The markedly basophilic growth protrudes on the pleural surface. It has filled several alveoli with compact masses of cells but its central portion is adenomatous. Other sections showed mitoses to be fairly numerous in it. $\times 294$.

FIG. 3. Adenoma in a 10-day-old sibling of the mouse furnishing Fig. 1. As in the case of the other early tumors there is no reactive proliferation or cellular accumulation about the growth. It looks as if it had originated from the epithelium of the bronchial tree, but serial sections showed it to be isolated amidst parenchyma. $\times 321$.



PLATE 25

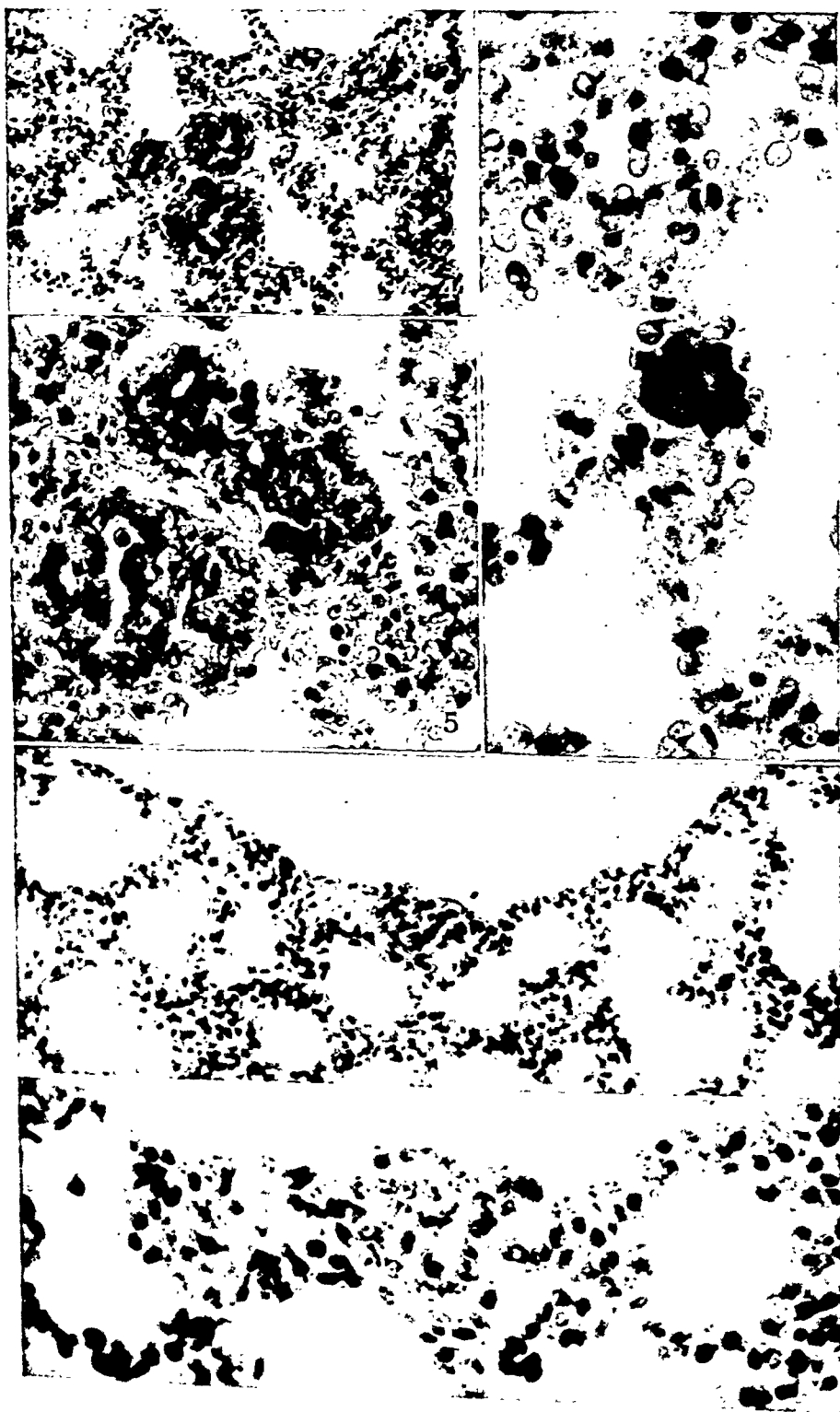
FIG. 4. Adenoma in a 3-day-old mouse born 7 days after the last of four urethane injections into the mother (mother IX of Table 2); total period since the first injection, 14 days. At the level here pictured there appear to be three separate islands of neoplastic proliferation, but they were parts of a single growth. It lay in the parenchyma far from any bronchiole and stained a much deeper blue than the epithelium lining the latter. $\times 186$.

FIG. 5. Higher magnification of the same tumor at another level,—to show its "glandular" arrangement and cuboidal cells. $\times 401$.

FIG. 6. Subpleural adenoma in a 3-day-old mouse born 2 days after the last of six injections of urethane into the mother (mother X of Table 2); total period since the first injection, 11 days. The growth is discrete. $\times 250$.

FIG. 7. The same tumor at higher magnification. It is of typically adenomatous character. There is no cellular reaction about it. $\times 569$.

FIG. 8. Clump of deeply basophilic cells with vesicular nuclei; lung of a 3-day-old litter mate of the animals providing the growths of Figs. 1, 3, 9, and 10. Similar clumps of cells have occasionally been seen in normal C strain mice 10 days old. $\times 569$.



Smith and Rous: Neoplastic potentialities of mouse embryonic tissues. IV

PLATE 26

FIG. 9. Adenoma in a 3-day-old litter mate of the animals furnishing Figs. 1, 3, and 8. The growth lies in the parenchyma and is of the compact type. It stained bright blue against the general pink. $\times 277$.

FIG. 10. Section through the edge of the growth of Fig. 9, where it covers part of an alveolar wall with a single layer of cells. The arrow points toward a mitosis; there were three in the tiny growth. $\times 527$.

FIGS. 11 and 12. Adenoma (?) in the lung of an embryo near term. The mother had received six injections of urethane, the last one 24 hours before the embryo was procured. The growth bulges on the pleural surface, appears to have some acinar arrangement, and its cells occupy part of a neighboring alveolus. The embryonic state of the pulmonary tissue is evident. Fig. 11, $\times 280$. Fig. 12, $\times 449$.

FIG. 13. Adenoma of the glandular type in a 10-day-old mouse from a mother of the A strain which had received three injections of urethane, the last within 24 hours of parturition; total interval since the first injection, 14 days. $\times 449$.

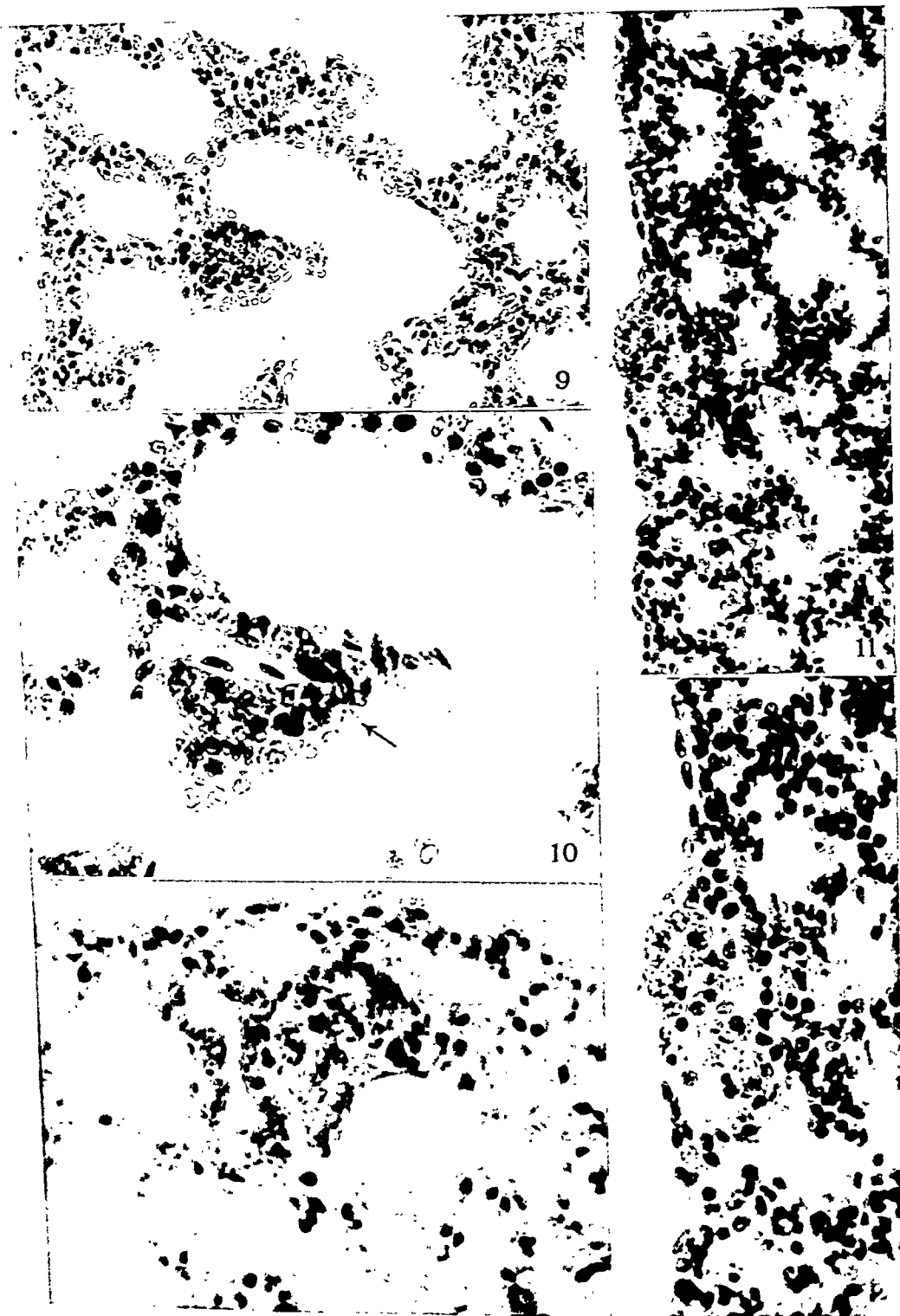


PLATE 27

FIG. 14. Another "glandular" adenoma from the same animal; there were only the two growths. The tumor is markedly basophilic, as in the previous instance. Some atelectasis is present owing to delay in fixation of the lung after it had been removed from the animal. $\times 422$.

FIG. 15. Compact adenoma in a 10-day-old mouse from another female of the A strain injected thrice with urethane, the last time within 24 hours of parturition. The growth shows the usual basophilia. The arrow indicates a mitotic figure. $\times 422$.

FIG. 16. Mooring clump with membrane attached near by; from a day-old mouse of a C strain mother. Serial sections showed the clump to be spindle-shaped and almost $300\ \mu$ long. The arrow points to a mitosis. $\times 422$.

FIG. 17. Peripheral zone of an adenoma, with part of an adjacent bronchiole, in a mouse killed 70 days after birth of her offspring. She had received three injections of urethane during pregnancy.

The growth differs considerably from those in sucklings. Interspersed among cells with round, vesicular nuclei, such as make up the tumors in these latter, are many with nuclei that are oval or oblong and almost pyknotic, often relatively large and sometimes with more cytoplasm about them than ordinary. In one spot near the lower edge of the picture nuclei of this sort lie with their long axes parallel, and the cells appear cylindrical, as if from lateral compression. The epithelium of the bronchiole has precisely the same general morphology as the component elements of the tumor except that some of its cells are more cylindrical. $\times 278$.

FIG. 18. Low magnification of the same tumor, to show where it has filled a space into which the bronchiole opens. Though its cells closely resemble the epithelium of this latter the two are not joined. At the center of the growth, where degeneration was under way, the cytoplasm had a blue cast on staining, but at the periphery it was almost as pink as the bronchiolar epithelium. $\times 136$.

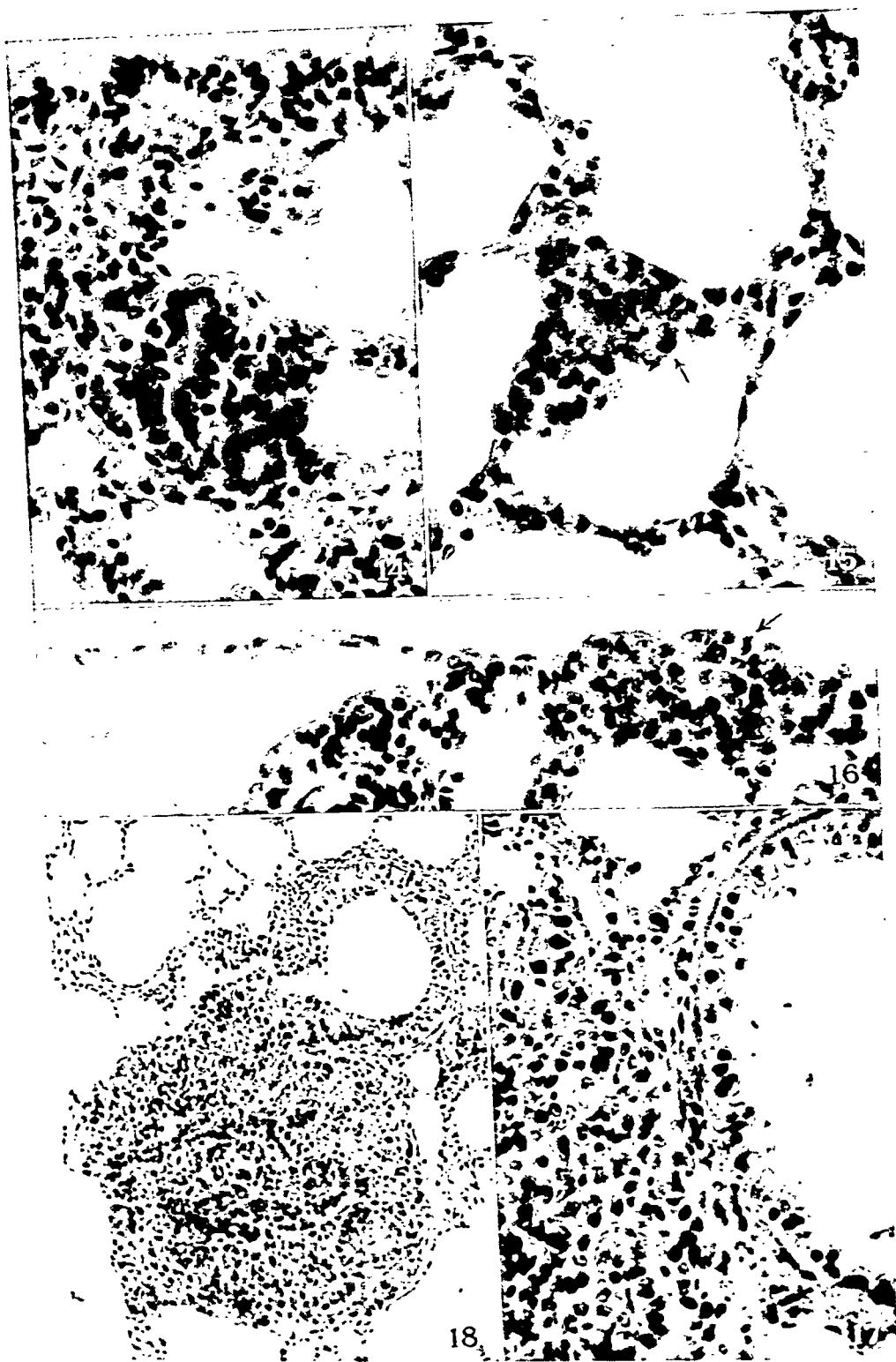


PLATE 28

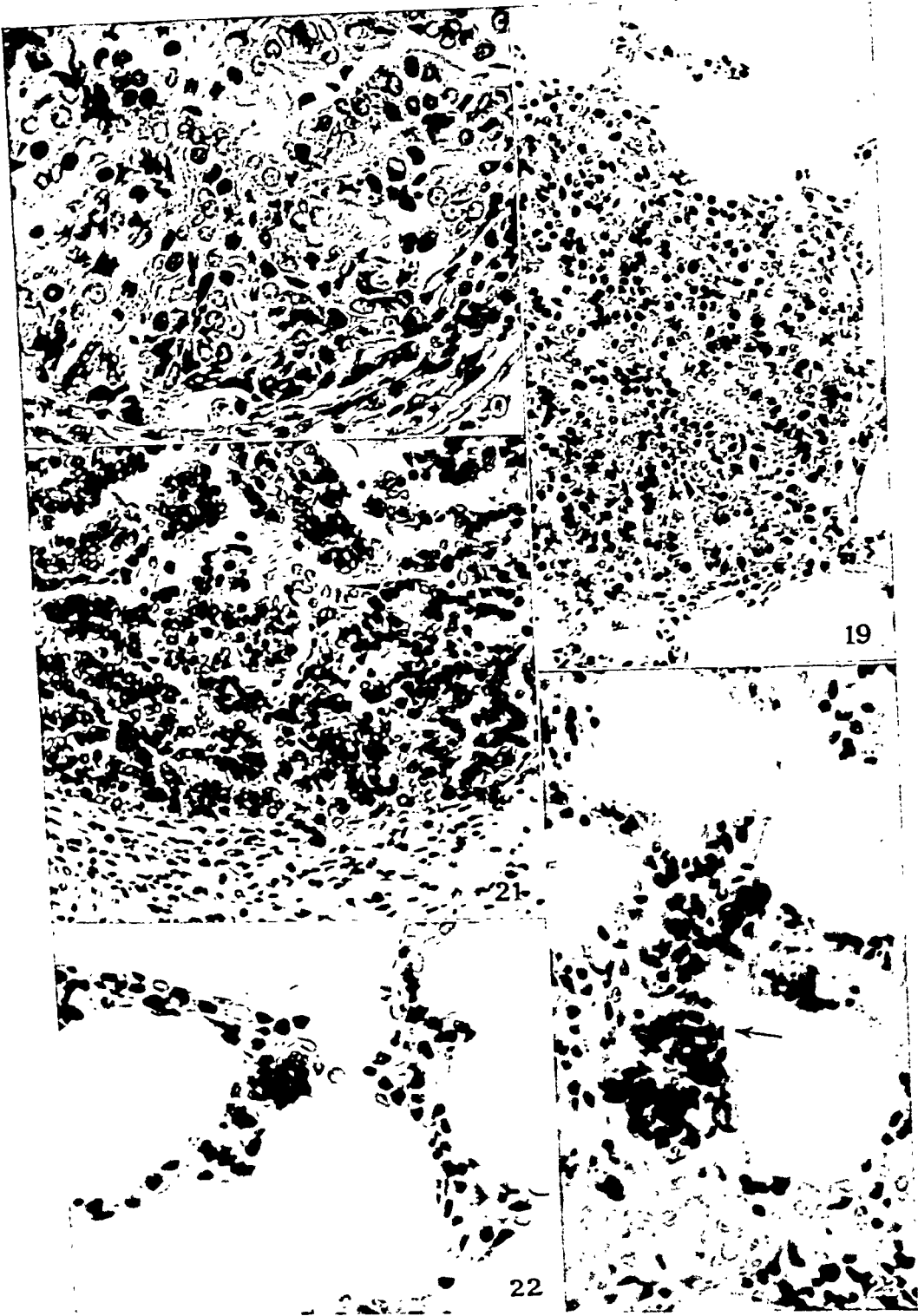
FIG. 19. Another adenoma from the same animal as that of Figs. 17 and 18 and with the same cytological features. It was not directly connected with any bronchiole. $\times 243$.

FIG. 20. To show the "bronchiolar" character of the cells of an adenoma found in an animal 70 days after birth from a urethanized mother (mother G of Table 3 and 4). Though unusually large (1.5 mm. in diameter) the growth was devoid of mitotic figures. The tissue next its border is compressed. $\times 449$.

FIG. 21. Edge of another exceptionally large adenoma (2 mm. across) in a 70-day-old mouse from a urethanized mother (mother A of Tables 3 and 4). The growth is markedly basophilic and differentiation is not as far advanced as in the tumor of Fig. 20 yet many dark-staining nuclei are present, of the sort found in mature adenomas. Mitotic figures were rare (Table 4). $\times 277$.

FIG. 22. Clump of cells in an alveolar wall of a normal 3-day-old mouse of the C strain. The cells are notably basophilic, as in the case of adenomas, but the nuclei were smaller than in these latter and were not vesicular. $\times 527$.

FIG. 23. Dubious growth in another 3-day-old control of the C strain. The cells are basophilic and at one spot they lie in two parallel rows (arrow), but many of the nuclei are oblong and they are unusually small. $\times 449$.



THE STRUCTURE OF HUMAN SKIN COLLAGEN AS STUDIED WITH THE ELECTRON MICROSCOPE*

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PLATES 29 TO 32

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The application of modern methods of structural analysis in the colloidal range of dimensions to the study of connective tissue is of importance not only for the information it may yield regarding the molecular organization of this tissue but also because it may provide a means of investigating pathological alterations of the various components. Thus far, normal and abnormal mesenchymal tissues have been investigated primarily by histological and cytochemical methods. These techniques, because of their limited resolution, permit observation of changes in the fiber as a whole but cannot localize the alterations to the component collagen fibrils¹ or the interfibrillar substance.

The development of electron microscopy now permits direct visualization of tissue components of the order of 50 Å in size and even smaller. However, before one can profitably investigate abnormalities it is necessary to determine the structure of normal connective tissue and to establish the range of normal variation. Two factors make this task difficult. The fragmentation techniques used to prepare specimens thin enough for electron microscopy produce certain alterations in the material which must be evaluated by studying many different samples. The samples themselves are minute, which makes a statistical approach necessary. Further improvements in methods of preparing thin sections for electron microscopy (9, 20) may minimize certain of these difficulties.

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[†] This work was done during the tenure of a Life Insurance Medical Research Fellowship.

¹ Various hierarchies of fibrous structures may be distinguished in collagen. At the level of the light microscope one sees fibers as thin as 2 μ , or, as in skin, as thick, as 20 to 40 μ . These fibers comprise bundles of still thinner units, the *fibrils*, which can be observed in teased preparations with the darkfield microscope or with the electron microscope. In certain forms of connective tissue the fibrils have fairly uniform widths (about 0.1 μ , or 1,000 Å in skin) while in other types there is considerable scatter in the widths. The fibrils are presumed to be composed of bundles of polypeptide chains. These bundles which, for convenience, are called *filaments* have variable widths, from about 100 Å to the limits of electron microscope resolution.

A beginning has been made in the investigation with electron microscopy of elastic tissue (29) and of one of the connective tissue polysaccharides, hyaluronic acid (10). Considerable attention has been devoted to the collagenous constituents. A periodically banded pattern was first demonstrated in electron micrographs of collagen fibrils in 1942 (11, 24). The distance between repeating bands (axial period) agrees well with that previously demonstrated by Bear (2, 3) and Kratky and Sekora (15) by x-ray diffraction methods. Wolpers (28, 30) observed the striated appearance in fibrils of osmic acid-fixed tendon, cartilage, and umbilical cord. He resolved two bands in the dense region of each period.² Using phosphotungstic acid (PTA) as an "electron stain," Schmitt, Hall, and Jakus (23; see also Schmitt, 22) resolved more intraperiod fine structure in rat tail tendon fibrils in the form of five bands having characteristic position and density. Six intraperiod bands were observed in mammalian skin collagen by Nutting and Borasky (19) and Schmitt and Gross (21), and as many as seven bands were observed in the axial period of fibrils in rat epineurium and kangaroo tendon (21). It seems probable that, as further technical improvements are made, still further detail of the axial repeating structure will be resolved.

Application of metal shadowing technique has demonstrated that the cross-bands represent periodic variations in thickness of the dried fibrils. Two intraperiod elevations were previously demonstrated (23, 19) but recently six elevations were resolved in platinum-shadowed fibrils of goat and calf skin, corresponding well with the relative positions of the bands in PTA-stained collagen (21).

In the present study a replica technique was used in order to permit examination of moist fibrils and to allow observation of the surface characteristics of tissue fragments too thick for transmission electron microscopy.

The primary purpose of this paper is to describe the structure observed in the collagen fibrils of normal human skin and to attempt to provide a baseline for reference in subsequent studies of collagen from pathological tissue.

Methods

Samples of abdominal skin were obtained at autopsy from 16 individuals varying in age from 1 hour to 89 years. None suffered from any known widespread disease of the connective tissue.

The unfixed skin was dissected free of readily removable fat and subcutaneous tissue, sectioned parallel to the surface with the freezing microtome, and the epidermis discarded. The frozen sections were placed in double distilled water and dispersed with needles or further fragmented in a microblendor or by sonic (9 kc.) treatment.

Some of the crude water suspensions were treated in the following manner: (a) Thorough washing with double distilled water in the clinical centrifuge. (b) Incubation in crude or crystalline trypsin (0.1 per cent in isotonic saline or distilled water) for 24 hours at 37°C. The suspension was then washed in double distilled water. (c) Incubation in hyaluronidase,³ in varying concentration up to 20 mg. per cent in water or isotonic saline at 37°C. for 2 hours, followed by washing.

² In a personal communication Wolpers described four bands in the dense part of the period.

³ This preparation of testicular hyaluronidase was kindly furnished by The Schering Corporation.

Specimens were prepared for electron microscopy by placing a drop of suspension on the usual collodion or formvar supporting film of the specimen grid. In a few cases frozen sections were smeared onto specimen films without preliminary fragmentation.

When staining was desired the specimen grid was drained and a droplet of PTA solution (0.1 to 1.0 per cent in phosphate buffer at pH = 5) applied. The grid was inverted, to avoid deposition of unwanted suspended material and staining allowed to proceed for periods varying from 15 seconds to 5 minutes. The preparation was then washed by immersion in double distilled water, with gentle agitation, for about 15 seconds and air-dried.

For metal shadowing, collagen fibrils were deposited on a film-covered grid, air-dried, and placed in an evaporation chamber where they were subjected to a beam of metal atoms impinging at angles varying from 6 to 20°, after the method of Williams and Wyckoff (26). Chromium or platinum were usually used; in the former case the calculated thickness of deposited metal was about 15 Å normal to the film, in the latter case 4 to 6 Å.⁴

The technique of preparing thin plastic films, described by Shaefer and Harker (25), was modified for use in preparing replicas. Various combinations of plastics and solvents were used. These included collodion or methyl methacrylate in secondary butyl acetate, formvar in ethylene dichloride, and polystyrene in benzene. A 0.2 per cent solution was found to provide optimum film thickness.

A water suspension of fragmented connective tissue was air-dried on a clean glass slide. With a very thin film of moisture still present on portions, the slide was plunged into the plastic solution, allowed to remain for 5 to 30 seconds, then dried in the vertical position in air. The moist patches could be seen beneath the dried plastic. The film was then sectioned into squares with a needle and floated off onto a clean water surface. These portions of film were then transferred to nickel grids and shadowed with chromium in the usual manner in order to increase contrast. Other glass slide preparations were completely desiccated in a vacuum chamber before applying the plastic. Frozen sections of tissue were smeared directly onto the glass slide and replicas prepared therefrom. A similar technique has been used for the study of bacterial and bacteriophage colonies on the surface of agar (7, 14). Claude (4) has also used the method for the examination of air-dried blood cells.

An RCA type EMB electron microscope was used in this study. All measurements of intraperiod band positions were made with a microdensitometer from the original electron micrographs.

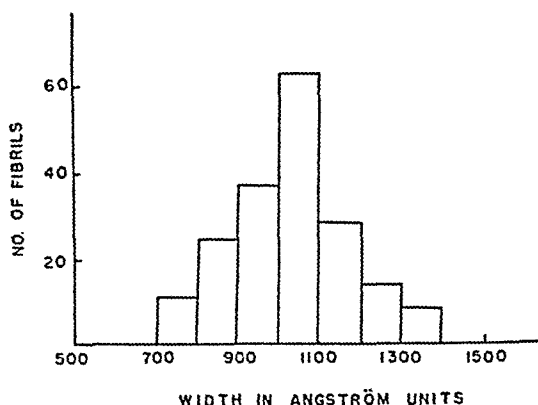
RESULTS

When frozen sections of human skin, cut parallel to the surface, are fragmented in water the solution becomes opalescent due to the release of collagen fibrils in various states of aggregation from single fibrils to bundles of fibrils. Viewed in the darkfield microscope the fibrils from adult skin appear as smooth, relatively straight, unbranched threads many microns in length. The fibrils of corium from infants are much more cohesive as judged by the amount of fragmentation required to disintegrate the tissue. In the darkfield these fibrils are more frequently clumped and appear to be finer, shorter, and more cortorted than are those of adult skin.

⁴ Although the high temperatures required for evaporation of platinum may affect the specimen, this metal is preferable to chromium because of the thinness of the layer necessary to give the desired contrast; moreover, it does not aggregate in the electron beam as does gold. Evaporation of platinum is facilitated by the addition of 1 part of palladium to 4 parts of platinum, as suggested by Williams and Bachus.

In Fig. 1 is shown an electron micrograph of a group of fibrils from an incompletely fragmented tissue section. In such bundles of fibrils the cross-bands are frequently in lateral register. It is probable that this type of organization is characteristic of intact, normal tissue.

Fibril Widths.—Limitations of resolution make it difficult to measure widths of individual fibrils with an accuracy greater than about 50 Å. However, the collagen fibrils of different forms of connective tissue vary considerably in range and uniformity of widths. In Text-fig. 1 is shown a distribution curve of widths measured on 180 fibrils of adult human skin. Widths range from about 700 to 1,400 Å, the majority being about 1,000 Å. Fibrils from infant skin show widths as small as 300 Å though the average is not significantly different from that of adult skin. Wolpers (27) stated that the fibrils are



TEXT-FIG. 1. Distribution of fibril widths (180 fibrils measured)

thinner in young than in old individuals. The widths of fibrils of rat tail tendon range from almost as small as can be resolved by the electron microscope to 2,000 Å and more (24). This variation probably reflects a real difference in the organization of the fibrils in different tissues rather than alterations due to the method of preparation.

Axial Repeating Period.—A distribution curve of values of the axial repeating period of skin fibrils was constructed. At least 10 consecutive periods in each of 200 chrome-shadowed fibrils were measured. As shown in Text-fig. 2, the periods ranged from about 500 to 800 Å, the maximum occurring between 620 and 660 Å. This distribution is similar to that found by Nutting and Borasky (19) in cow skin fibrils; the range of variation is somewhat less than that found for the collagen of rat tail tendon by Schmitt, Hall, and Jakus (24). The average period in fibrils of adult skin does not appear to differ significantly from that of fibrils from infant skin.

Axial Intraprimer Structure.—Unstained skin fibrils usually show little detailed intraperiod structure but resemble that shown in Fig. 3. In rare in-

stances as many as five intraperiod bands were distinguished. It is possible that, with different technique, fine structure may be resolved with more regularity. However at present, staining is required to bring out detailed fine structure in most preparations.

Even after staining with PTA the fraction of fibrils showing more than two or three dense bands per period is small. This is probably due not to a deficiency in the resolving power of the electron microscope but rather to imperfections of organization of the fibrils resulting from distortions introduced during preparation of the specimen. Wolpers (27) believed that removal of ground substance by sonic treatment is required for visualization of fine structure. However, in the present studies, the highest proportion of fibrils showing detailed intraperiod fine structure was found in a specimen in which the fibrils were embedded in an amorphous matrix which could not be removed by water washing. While such amorphous material undoubtedly reduces resolution of fibril structure, the primary factor in observing fine structure is probably distortion of the lateral relationship of the filaments within the fibril.

The most characteristic intraperiod band pattern observed in well preserved fibrils is that containing six unequally spaced bands as shown in Fig. 5. This pattern has been observed in fibrils of animal skins, tendon, and nerve epineurium (21). The bands have been tentatively termed: *a*, *b*₁, *b*₂, *c*, *d*, and *e*. The *a* band, which is located at the widest part of the period in cases where the fibril shows a scalloped appearance, is the broadest and most dense of the six bands. In a few cases the *a* band appeared as a doublet. The adjacent *b*₁ and *b*₂ bands have the smallest separation; with poorer resolution these appear as a single band, *b*, as previously observed in rat tail tendon fibrils (22).⁵ Fig. 4 illustrates this pattern in a skin fibril stained with 0.1 per cent PTA. The *d* band is characterized by its relatively high density in contrast to adjacent regions. The *c* band is not sharply defined, probably because it represents several unresolved, fine bands; two of these subdivisions were observed in a somewhat stretched kangaroo tendon fibril. The *e* band is almost always diffuse and of relatively low density.

The average separation between bands, is about 18 per cent of the total period except for the *b*₁-*b*₂ separation which is about 11 per cent. These values agree well with those observed in fibrils of chrome-tanned calf and goat skin by Schmitt and Gross (21). They are essentially constant in the range of periods for which measurements were made (500 to 900 Å).

It is probable that, as improvements are made in methods of preparation of the material and in the imaging ability of the electron microscope, still further

⁵ There is some indication that conditions of staining may be important in resolving these bands. Successful resolution occurred when 1 per cent PTA was used whereas, when the PTA concentration was 0.1 per cent, only one band in this region (6) was observed. There is a possibility, however, that the five-banded pattern is an actual entity and represents another form of collagen.

details of intraperiod structure will be resolved. The present observations were made with the through-focus technique; *i.e.*, a series of micrographs was made of each specimen at different focal levels. This not only facilitates obtaining the best definition but also permits evaluation of diffraction effects characteristic of such finely banded structures.

Chromium- or platinum-shadowed fibrils show two characteristic intraperiod elevations as a rule. However, additional fine structure is frequently visible and, in the most favorable cases, six intraperiod elevations are seen (Fig. 7). These elevations correspond in position with the six bands observed in stained fibrils.⁶

From this evidence it appears that the cross-striations are regions which are relatively thicker than the interband regions. This suggests that the contrast produced by staining with heavy metal salts could be due in part to a volume effect as well as to a specific chemical combination of stain with groupings in the collagen molecules. Nutting and Borasky (19) favored the former suggestion.

Lateral Structure in Fibrils

It has been shown that the collagen fibril is composed of very thin filaments of varying widths ranging from about 100 Angström units down to and below the limits of resolution of the electron microscope. The filaments have been directly visualized in acetic acid "solutions" of rat tail tendon and fish swim bladder ichthyocol.⁷

In shadowed human skin fibrils, particularly when the beam of metal atoms impinges on the fibril at right angles to the fibril axis, a fine longitudinal filamentous structure can be observed (Fig. 8). From three to ten such filaments may be visible in different regions of the same fibril. The filaments pursue a predominantly longitudinal course and frequently may be followed individually through a number of periods. Where they intersect the cross-bands there is a nodose appearance.

Such filaments may well be aggregates depending on factors involved in preparation, chiefly those operating during drying. However, they probably represent potential cleavage planes. In rat tail tendon the filamentous nature of the fibril is readily demonstrated because of the tendency of these fibrils to cleave longitudinally when fragmented. Skin fibrils seldom show such cleavage; initial fracture usually occurs in directions roughly normal to the axis. A fraction of the observed severed ends represents fibrils which were cut by the microtome. However, we have observed many cases such as that illustrated in Fig. 9. Here may be seen the fractured end of a fibril with the replica imprint of the lost fragment, showing that the fracture occurred after the fibril

⁶ While it is difficult to reproduce such electron micrographs, the positions of the bands are readily determined with the microdensitometer.

⁷ Unpublished data.

was deposited on the slide. Occasional disruption of a fibril has been observed but no true fray has been seen. The disorganized regions appear amorphous instead of filamentous as does rat tail tendon.

In a number of micrographs there have been observed ellipsoidal, disc-like objects having diameters approximately equal to those of the fibrils. It is possible that these represent thin sections of fibrils, possibly one or several of the repeating periods, removed during fragmentation. This idea is supported by the fact that, in occasional instances, entire periods were missing along the lengths of fibrils, or were seen to be displaced from the fibril axis.

Where the fibril adheres firmly to the plastic film it may flatten to a thin ribbon on drying (Figs. 6 and 9). Nevertheless, the axial repeating pattern is usually as well preserved in such flattened fibrils as in those which dry with a more circular or elliptical cross-section. Apparently the filaments which compose the moist fibril are relatively free to rotate about each other without loss of regular axial repeating structure and whilst maintaining considerable interaction with each other laterally, so that the fibril is uniformly cross-striated.

Information about drying patterns is given also by shadowed specimens. In Fig. 7 is seen a fibril shadowed at an angle oblique to the fibril axis. The wedge-shaped shadow at the lower left region of each period demonstrates the cylindrical character of the fibril. When the fibril flattens to a ribbon on drying this feature is not present and shadows are very short or absent. The ribbon-like character of the fibril in Fig. 6 is further indicated by the wrinkling at the right end, produced by bending in a direction parallel to its flat surface.

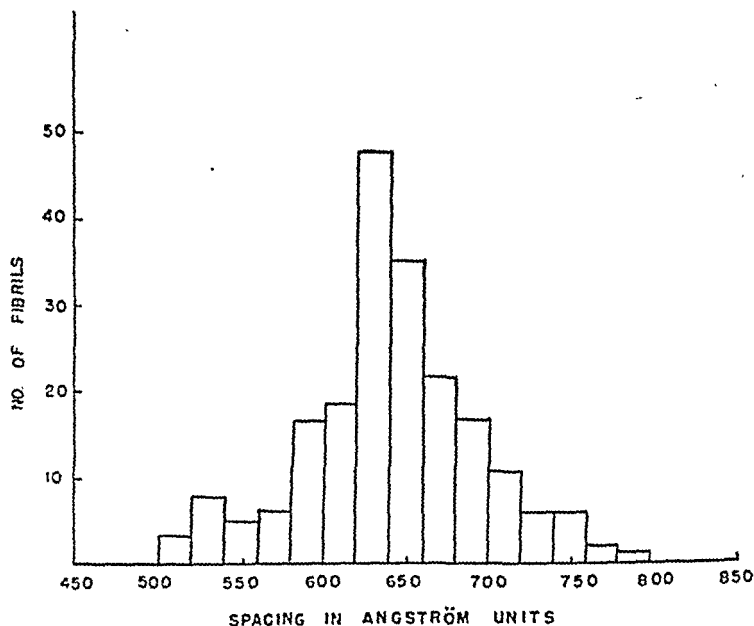
Replica Studies

Replicas of Dried Fibrils.—In the preparation of replicas of collagen fibrils deposited on glass slides many of the fibrils are removed with the plastic replica film. These may be found embedded in the plastic film or somewhat displaced from their imprints as a result of tensions during stripping (Fig. 2). This fortunate circumstance permits comparison of the structure of the dried fibril with that of its imprint in the replica. In the majority of cases the fibrils remain on the glass slide and only their imprints are seen.

The axial repeating period is well shown in replicas. The distribution curve of 100 imprints resembles closely that obtained directly from the fibrils (Text-fig. 2). The best replicas show two and three intraperiod bands (Fig. 11) but the definition is not good enough to permit measurement of positions. In rare cases four bands may be seen.

The elevations in the replicas represent depressions (thin regions) in the collagen fibril. This information about the contour of the intraperiod bands of dried fibrils supplements that obtained from the shadowed fibrils themselves. This contouring is best observed when the metal atoms fall upon the replica in a direction parallel with the fibril axis during shadowing.

The solvent used for dissolving the plastic may be of importance in this technique. The replicas of some dried fibrils prepared with ethylene dichloride as solvent for formvar were considerably wider than the fibrils themselves and in many cases little axial repeating structure was shown. The possibility that the solvent may have reacted with the collagen in such instances is being fur-



TEXT-FIG. 2. Distribution of axial periodicity (200 fibrils measured)

ther investigated. Fig. 2 represents an unusually good formvar replica of dried fibrils. Collodion in sec-butyl acetate has proved most satisfactory to date. Fig. 10 is a good example of a dried collodion replica of collagen bundles.

Replicas of Moist Fibrils.—It is clear from x-ray studies (3) that drying alters the structure of collagen in a characteristic fashion. Electron microscopy of collagen fibrils is possible only in the dried state. However, information concerning the structure prior to drying may be obtained from replicas of moist fibrils.

Replicas of moist fibrils resemble impressions made in smooth sand.⁸ In Fig. 12 (collodion replica) two crossed fibrils were transferred from the glass with the film. A faint periodic contouring, corresponding to the axial repeating pattern, is usually seen in the imprints. However, in some cases this is almost completely lacking, indicating that the surface of the moist fibril may be essentially smooth.

The widths of replicas of moist fibrils are usually somewhat greater than those

⁸ In our hands formvar in ethylene dichloride produces unsatisfactory, coarse-grained replicas of moist specimens, probably because of poor spreading on wet surfaces.

of the fibrils themselves and the edges are poorly defined. This may be due to the tendency of the moist fibrils to flatten and adhere firmly to the glass surface.

Replicas were readily prepared from smears made by gently rubbing fresh tissue sections on a clean glass surface. Fig. 13 shows a replica of a group of fibrils obtained in this manner. The axial repeating pattern is well represented as are some details of structure. Apparently these fibrils were aligned with periods in lateral register. Similar micrographs have been obtained from the skin of newborn as well as aged individuals. This technique, which is readily applicable to biopsy specimens, is particularly useful in studying the relationship of the various tissue components and is thus a valuable supplement to methods of fragmentation and sectioning.

In all aqueous suspensions of fragmented connective tissue considerable amounts of amorphous material were present, the largest quantity occurring in infant skin where it almost completely obscured the axial periods of the collagen fibrils. Even after thorough washing in double distilled water this material persisted to a greater or lesser degree in most specimens. Replicas of smears made directly from the frozen sections of both adult and newborn infant skin produced imprints of fibrils which showed about as much detailed axial structure as did those of well washed collagen (Fig. 13). This suggests that some at least of the fibrils in the intact adult and infant corium are not closely invested with a layer of adherent interfibrillar ground substance. Smears of the fresh tissue made directly onto the supporting film of the specimen grid and shadowed with chromium were usually too thick for satisfactory study. However in a few instances they showed some detail of structure (Fig. 14). These fibrils were embedded in considerable amounts of apparently structureless material, although their free surfaces were relatively clean.

Miscellaneous Observations

In preliminary experiments no action of trypsin and hyaluronidase on the collagen of infant and adult skin was observed. However, different methods will have to be devised to investigate the action of enzymes, particularly on the amorphous matrix, because the products of the reaction cannot be differentiated from other amorphous material usually found in such preparations.

In all the preparations of connective tissue studied in these experiments the paucity of formed constituents other than collagen fibrils and poorly defined amorphous material was noteworthy.

Occasionally structures resembling elastic tissue elements, as described by Wolpers (29), were observed.

Although histological examination of infant skin, from the same specimens studied with the electron microscope, showed argyrophilic fibers characteristic of reticulin, no differentiation between collagen and reticulin fibers was obvious in the electron microscope.

In some specimens of infant skin occasional smooth edged filaments, about 100 Å in width and of variable length, were noted. These occurred as individual filaments and as bundles of filaments. Their nature is at present not clear.

One grid, prepared from a shadowed replica of fragmented skin, bore no typical collagen fibrils but showed numerous, broad, double edged, striated fibrils surrounded by dense, adherent amorphous material. Except for considerably greater widths, these fibrils are suggestive of the "neurotubules" described by De Robertis and Schmitt (6). Possibly these structures derived from cutaneous nerves or their terminal twigs.

DISCUSSION

From the results obtained with replicas of moist fibrils it appears that the collagen fibril, as it exists *in vivo* is essentially a smooth contoured cylinder or ellipsoidal ribbon. Removal of water, in drying, gives the fibril a contour whose details conform to those of the banded structure seen in stained or shadowed fibrils, possibly resulting from differential shrinkage of highly localized regions within the repeating period. Obviously the structure observed in any particular collagen fibril, whether from normal or abnormal tissue, depends upon the internal adjustments of the fibril during the processes of tissue fragmentation, staining or shadowing and, finally, a very thorough drying. This internal adjustment probably concerns the interrelationship of the filaments thought to compose the fibril. It has been emphasized above that the range of variability of intraperiod fine structure observed in fibrils from one particular normal tissue, the collagen of human skin, is great. It is possible that most of these variations depend on alterations produced by the preparative procedure, as mentioned above. However, it is also possible that inherent structural variations exist, not only as a function of type of connective tissue and of age, but also within closely delimited regions of the same tissue. In this case the difficulties of the electron microscope approach to the problem become greatly multiplied because of the necessity of sampling many regions of the specimen. This is true in respect to the details of structure which require the highest available resolution. Possibly valuable information will come, at lower resolutions, from examination of thin sections of connective tissue prepared by improved microtome techniques.

With these facts in mind it may be useful to indicate the detailed structure which appears best to characterize the collagen fibrils of normal human skin, as thus far investigated.

A large axial repeating pattern in the form of cross-bands is a characteristic shared with other fibrous proteins such as fibrin (13), keratin (8), paramyosin (12), and "neurotubules" (6). Nor is the range of spacings (500 to 800 Å in the present material) unique to collagen; it is essentially similar to that of neurotubules. The particular six banded pattern shown in Figs. 5 and 7 is,

so far as we know, characteristic of collagen, having been found in skin, kangaroo tendon, and rat epineurium. It is possible that, as more detailed intraperiod structure is resolved, the "collagen pattern" will be defined more exactly.

It should be pointed out here that, as Astbury (1) has shown from x-ray results, there is probably a class of collagen-like proteins, all having the property of manifesting the wide-angle x-ray pattern characteristic of vertebrate collagen. These proteins differ markedly in amino acid composition from vertebrate collagen and are obtained from animals as low in the phylogenetic series as the sponges. They also have an axial repeating period similar to that of vertebrate collagen though there are differences of intraperiod structure.⁹ Accordingly, it is possible that any protein belonging to the collagen class will show a banded structure in electron micrographs and that identification will depend on differences of detail.

Collagens differ also in respect to properties of the fibrils in the lateral direction. As brought out in the present experiments, the fibrils of skin do not show the longitudinal cleavage characteristic of tendon fibrils; mechanical fracture usually occurs normal to the axis. This suggests that the lateral bonding between filaments in the fibril may be considerably stronger in skin than in tendon fibrils (16). Strong lateral bonds between the main chains, contributed by the protein itself or by other substances, could produce a great increase in lateral cohesion. Careful quantitative studies of the strength of the various linkages in collagen are lacking. Such information might provide criteria as valuable for the characterization of collagen as structural data.

There is some evidence of lateral affinity between adjacent fibrils. It has been frequently observed, in bundles of closely adherent fibrils, that the fibrils are in nearly perfect register with respect to the cross-bands. Whether this is due to specific affinities of chemical groups at various levels in the periods or to some undefined binding material is not clear. It seems probable that this interfibrillar integration may play an important rôle in the mechanical properties of connective tissue.

In current studies it has been assumed that the fibrils possessing the structural characteristics described above are primarily composed of the protein collagen. It has been suggested that collagen is closely associated with other components such as polysaccharide (17) and protein (5). In the present experiments efforts have been made to test this view but the results are inconclusive. No clear-cut effects have been observed as a result of exposing skin collagen fibrils to the action of hyaluronidase. However, certain polysaccharides are insensitive to the testicular hyaluronidase used; if such be present, bound to the collagen as Meyer (18) suggests, no positive result would be expected in the above experiment.

Similar considerations apply to the experiments involving the effect of trypsin. This enzyme has been used frequently to rid preparations of non-

⁹ From unpublished data of M. H. Marks, R. S. Bear, and C. H. Blake.

collagenous proteins and thus better to reveal the structure of the collagen. The usual detailed fine structure has been observed in such enzyme-treated material. However, this is not proof of the absence of a non-collagenous protein in the fibril nor yet of the complete insensitivity of all collagen to tryptic attack. Steric effects and mode of packing might be responsible for the insensitivity. The subject of the action of proteolytic enzymes on collagen is a large and important one which warrants detailed study.

The present experiments throw little light on the nature of the amorphous ground substance in which the collagen fibrils are thought to be embedded in connective tissue. While purified hyaluronate has a characteristic morphology (10), this is closely dependent on methods of preparation and might well be masked in the present experiments, which involved extensive fragmentation and water washing. Possibly the replica-smear technique will prove valuable in the study of the amorphous component.

SUMMARY

1. The structure of the collagen fibrils of normal human skin corium has been investigated with the electron microscope.

2. Under the conditions of observation the fibrils ranged in width from about 700 to 1,400 Å with 1,000 Å as the value occurring most frequently. They showed little tendency to fray longitudinally as is characteristic of tendon fibrils; when fracture of fibrils occurred it was usually in planes transverse to the axis.

3. The axial repeating periods observed in fibrils stained with phosphotungstic acid or shadowed with chromium or platinum range from about 500 to 800 Å, the maximum occurring between 620 and 660 Å. The average period in fibrils from infant skin does not differ significantly from that of adult and aged skin.

4. Depending on conditions of preparation, intraperiod fine structure, in the form of cross-bands, was observed in varying detail. The most detailed pattern commonly observed contains six bands of characteristic density and position.

5. Shadowed plastic replicas of dried collagen fibrils reproduce much of the structure commonly seen in shadowed fibrils. Replicas of moist fibrils show considerably less surface contouring than do dried fibrils. Replicas from smears of connective tissue fragments on glass show detailed structure, indicating the feasibility of applying this technique to biopsy material.

6. Infant skin differs from adult skin in having considerably greater amounts of amorphous material, little of which is strongly adherent to the collagen fibrils.

Addendum.—Since submission of this manuscript we have received, through the courtesy of Dr. C. Wolpers, two papers by this author, not previously available to us (*J. Makromol. Chem.*, 1948, 2, 37; *Biochem. Z.*, 1948, 318, 373). Working chiefly with osmic acid fixed beef tendon, Wolpers studied the effect of thermal shrinkage and of acid treatment, as well as certain pathological conditions (rabbit virus myxoma) upon the detailed structure of the collagen fibril. The normal fibril, he finds, is characterized by the presence of two dense (δ) bands per period. After heating or other treatment four bands (δ lamellae) are observed. In the transformation from two to four bands he suggests that a cementing material ("*Kittsubstanz*") of unknown nature

is removed and "δ" particles make their appearance in the fine bands. Further heat treatment destroys the banded structure and leads to an irregular filamentous network which, when fully dissociated, produces glue or gelatin.

Wolpers' view that detailed intraperiod structure (more than two bands) is the result of alteration or degradation of the structure of normal collagen is in contrast to that expressed in the present paper, in which it is supposed that the normal fibril is possessed of very detailed intraperiod structure which may not be fully resolved because of various factors operative during the preparative procedure. In this connection it may be pointed out that detailed intraperiod structure (six bands) has been observed in material which had been subjected only to teasing with needles and brief water washing, followed by PTA staining. It seems improbable that such mild treatment could have caused the alterations visualized by Wolpers as responsible for the production of the multibanded structure.

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EXPLANATION OF PLATES

PLATE 29

Formvar replica of collagen fibrils from adult human corium deposited on a glass slide from aqueous suspension and air-dried. Shadowed with chromium.

FIG. 1. Fiber bundles adherent to film. In background a few imprints are seen. $\times 19,300$.

FIG. 2. Individual adherent and embedded fibrils and imprints. $\times 26,000$.

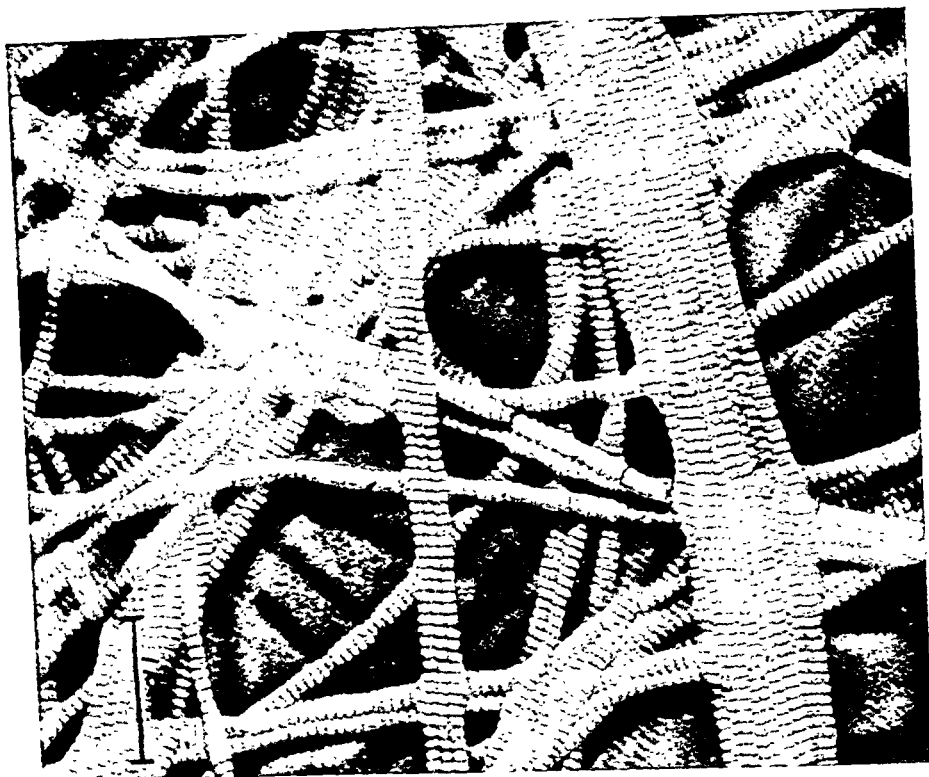


PLATE 30

Adult corium fibrils from fragmented frozen sections washed thoroughly in water and deposited on a specimen grid from suspension.

FIG. 3. Stained with 0.1 per cent PTA. Most unstained and many stained fibrils have this appearance in which little or no intraperiod structure is present. $\times 95,000$.

FIG. 4. Stained with 0.1 per cent PTA. Five bands resolved. $\times 100,000$.

FIG. 5. Stained with 1.0 per cent PTA. Six bands resolved. $\times 100,000$.

FIG. 6. Flattened fibril showing four and in some periods five bands. Note kinking of ribbon at right end. Shadowed with chromium. $\times 106,000$. (Arrow indicates direction of shadow.)

FIG. 7. Fibril showing six-banded period. Shadowed with chromium. $\times 80,100$. (Arrow indicates direction of shadow.)

FIG. 8. Longitudinal aggregates of filaments are seen in this fibril shadowed with chromium. $\times 98,000$. (Arrow indicates direction of shadow.)

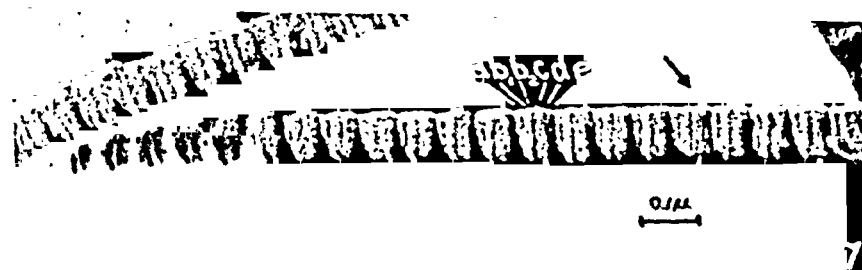
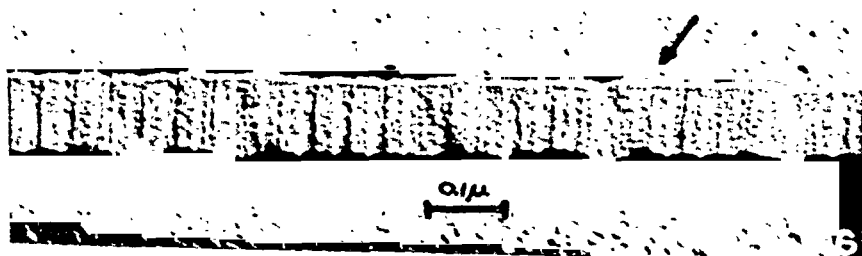
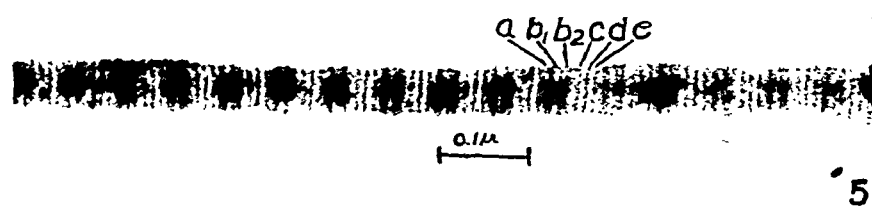
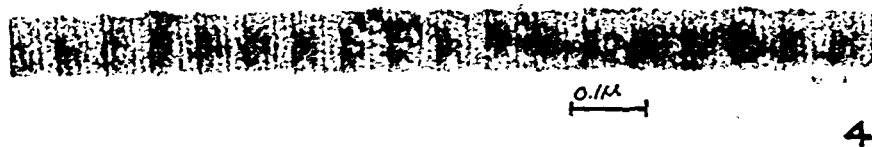
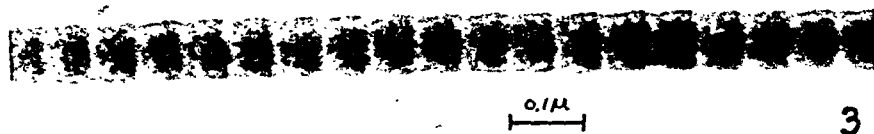
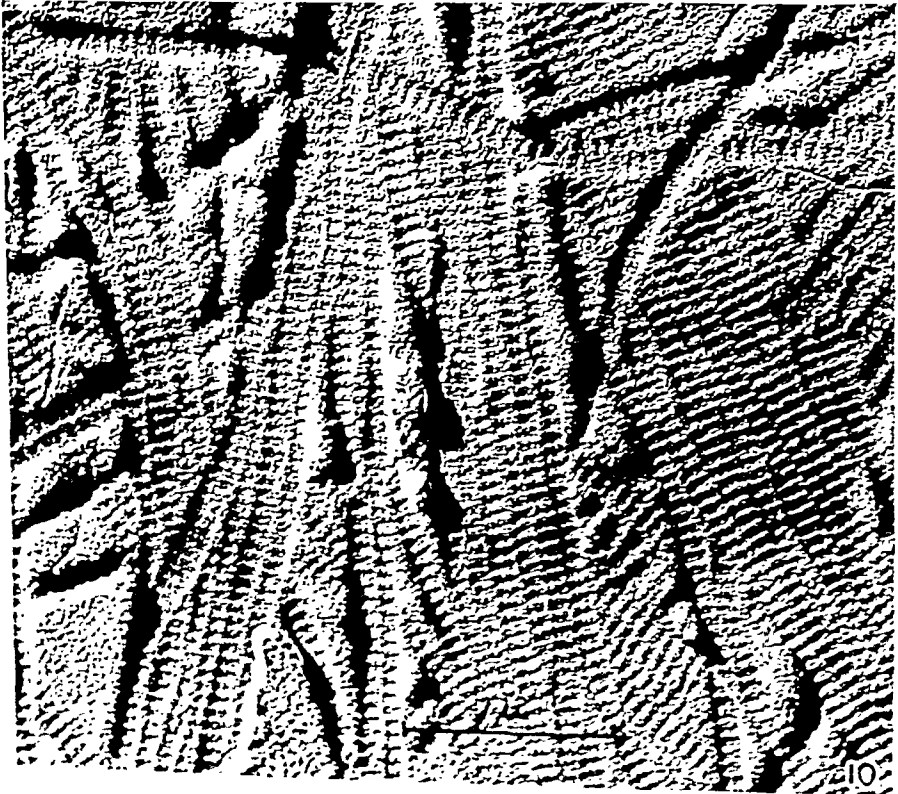
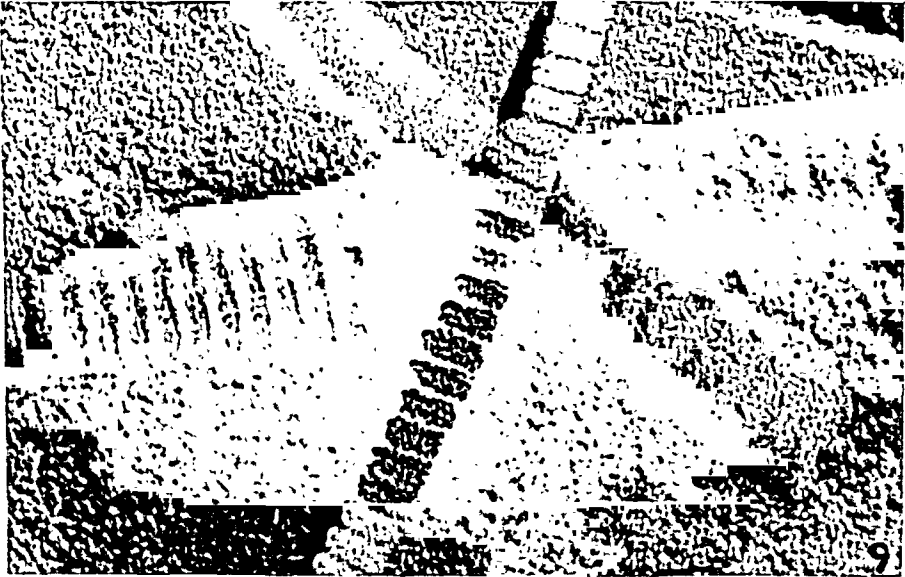


PLATE 31

FIG. 9. Chromium-shadowed formvar replica of air-dried fibrils deposited from suspension on glass slide. Note ruptured fibril at left with imprint of remainder; two other adherent fibrils are shown. $\times 61,500$.

FIG. 10. Collodion replica of vacuum-dried fibril bundles. Deposited on glass slide from water suspension. Shadowed with chromium. $\times 28,500$.



(Gross and Schmitt: Human skin collagen studied with electron microscope)

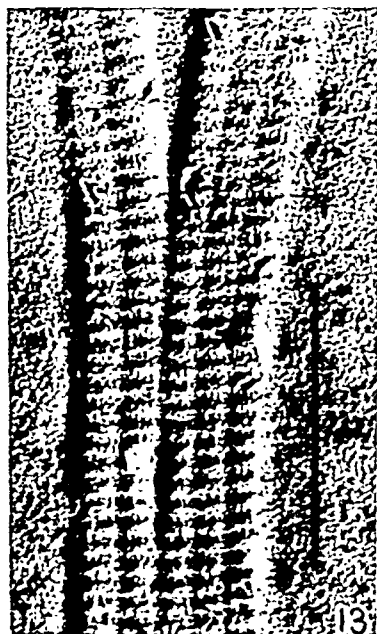
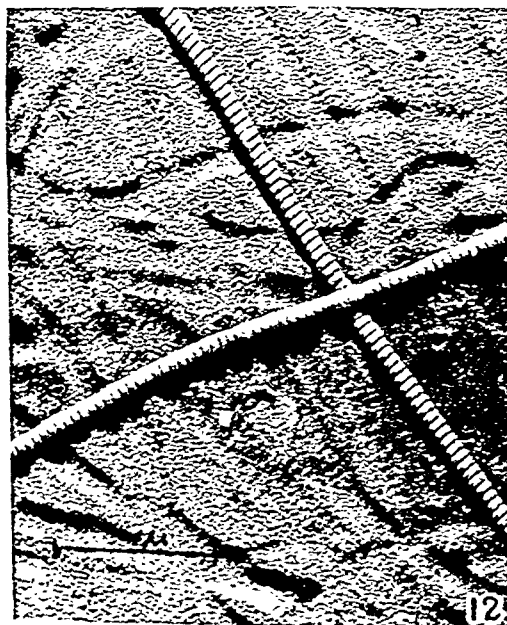
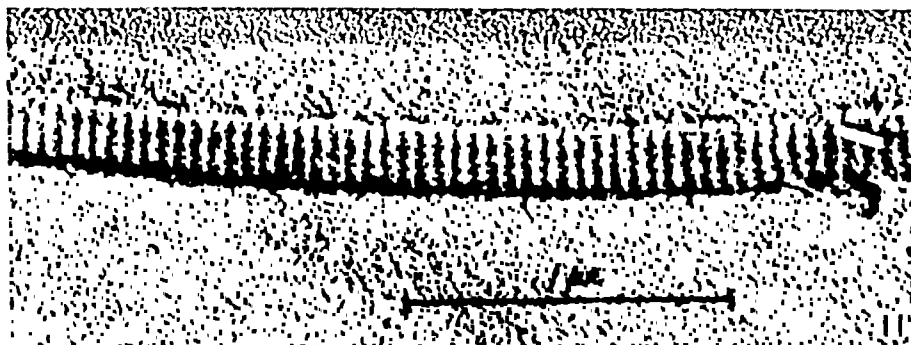
PLATE 32

FIG. 11. Chromium-shadowed collodion replica of air-dried fibril showing three intraperiod bands. Deposited on glass slide from water suspension. $\times 42,000$.

FIG. 12. Chromium-shadowed collodion replica of moist fibrils deposited on glass slide from suspension. Two adherent fibrils are present. Very little periodic structure is noted in fibril imprints. $\times 22,000$.

FIG. 13. Chromium-shadowed collodion replica of frozen section of adult human corium smeared directly onto glass slide. Showing imprints of a group of fibrils whose periods are in lateral register. $\times 36,000$.

FIG. 14. Direct smear of the corium of a 2-day-old infant onto supporting film of specimen grid shadowed with chromium. Axial periodicity can be observed despite the presence of surrounding amorphous material. $\times 27,000$.



(Gross and Schmidt. Human skin collagen studied with electron microscope)

THE INHIBITION OF *d*-GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE BY SPECIFIC ANTISERUM

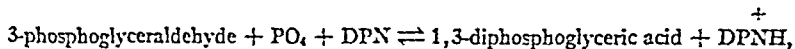
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(Received for publication, August 27, 1948)

This study was undertaken to determine whether *d*-glyceraldehyde 3-phosphate dehydrogenase from bakers' yeast and rabbit skeletal muscle are immunologically similar. Two such enzymes from widely separated sources might still be expected to have similar surface configurations in the region of the catalytically active centers, and this site might be involved in antibody formation. Evidence that antibody formation may be directed toward the active center of an enzyme was found by Kubowitz and Ott (1) and more recently by Zamecnik and Lipmann (2).

Antiserum to the yeast enzyme has been obtained from rabbits (3) and, as reported in this paper, from chickens. With one exception, the immune sera had no effect on the activity of the muscle enzyme in a test system in which the yeast enzyme was almost completely inhibited. The present report is a more detailed account of these investigations and an extension of the study to include experiments on the mechanism of inhibition of the yeast enzyme. Diphosphopyridine nucleotide (DPN) was found to protect this enzyme partially from antibody. It can be seen that in the reaction catalyzed by the dehydrogenase,



DPN is one of the reactants which combines reversibly with the active center of the enzyme.

EXPERIMENTAL

Enzyme Preparations.—(a) Yeast¹ *d*-glyceraldehyde 3-phosphate dehydrogenase was prepared by the method of Warburg and Christian (4) with some modification. With the yeast used, the yield of dry acetone powder obtained from the *Lebedev-Saft* (yeast juice) was

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† Senior Fellow, National Research Council. Aided by a Grant from the National Foundation for Infantile Paralysis.

¹ The authors wish to thank the Anheuser-Busch yeast plant for supplying the fresh starch-free bakers' yeast used in this preparation. We also are grateful to Sharp and Dohme, Inc., and the Eli Lilly and Co. for supplying us with samples of salmine sulfate.

only about one-half that reported. The nucleic acid-protein precipitate obtained between pH 5.0 and 4.5 was used instead of the fraction from 4.75 to 4.5. (This modification was found advisable also by Meyerhof and Junowicz-Kocholaty, using American yeast (5).) Salmine was used instead of sturine for the removal of nucleic acids, but was not added in amounts sufficient to remove the last traces of material absorbing at 260 $m\mu$. The $Al(OH)_3$ adsorption step was omitted, as this procedure was not found to increase the purity of the enzyme. The final protein solution was not concentrated by evaporation before the crystallization step. Solid ammonium sulfate is simply added to the 2 to 3 per cent protein solution at 25° until a faint turbidity develops. The suspension becomes crystalline after several days' storage at 0°. Crystallization is more rapid and complete if the pH of the suspension is raised to 8 with dilute NH_4OH solution. If microscopic observation shows a background of amorphous protein, warming the crystal suspension to 35° and slowly cooling it removes the non-crystalline material. Recrystallizations are readily carried out by dissolving the centrifuged crystals in water to give a 2 to 3 per cent protein solution and adding ammonium sulfate to incipient turbidity.

As reported by Warburg and Christian, it was found that the highly purified solution obtained immediately preceding the crystallization step had almost the same specific activity as the crystalline enzyme. Electrophoretic studies have shown, however, that several protein components are present. Crystallization results in the separation of a single component, but this is not reflected by any increase in activity (6). In most instances the nearly electrophoretically homogeneous crystals were used, but some of the animals received injections of the enzyme solution immediately before crystallization.

The crystalline enzyme prepared with the above modifications was found to have a somewhat different absorption curve in the ultraviolet than that reported (4). This is due to the presence of DPN (6) apparently bound in a similar manner to that found in rabbit muscle *d*-glyceraldehyde 3-phosphate dehydrogenase (7). A second difference noted with this enzyme preparation is the necessity of having cysteine present to obtain maximum activity.

(b) Crystalline rabbit muscle *d*-glyceraldehyde 3-phosphate dehydrogenase was prepared by the method of Cori *et al.* (8).

(c) Crystalline yeast hexokinase was prepared by the method of Berger *et al.* (9) from the same strain of yeast from which the dehydrogenase was isolated.

Immunization Procedure.—Four medium sized rabbits were given a series of five subcutaneous injections of 8 mg. of yeast *d*-glyceraldehyde 3-phosphate dehydrogenase at 3 day intervals. The crystalline enzyme, stored as a suspension of crystals in alkaline ammonium sulfate solution, was dissolved in water and injected immediately. Sera were obtained 2 weeks after the last injections. Stronger antisera were obtained after a second series of injections 2 months later. Two rabbits received as many as four series of injections.

Four young chickens (6-month-old pullets) were given a similar series of injections of the same enzyme intravenously. Following the third or fourth injection all the chickens showed signs suggesting anaphylaxis (blanching of the comb and staggering). One died immediately following the third injection. Sera were again obtained 2 weeks after the last injection.

Detection of Antibody.—Antibody levels of the sera were measured (1) by the precipitin reaction, using the conventional ring test with serial dilution of a 1.5 per cent solution of crystalline enzyme in saline, and (2) by the inhibition of activity of a given amount of yeast *d*-glyceraldehyde 3-phosphate dehydrogenase.

Enzyme activity was determined by following the reduction of DPN spectrophotometrically at 340 $m\mu$ (4). Crystalline yeast enzyme was dissolved in 0.006 M cysteine-0.03 M sodium pyrophosphate, pH 8.5, and diluted in the same buffer. Protein determinations on these solutions were made by the biuret method of Robinson and Hogden (10). 0.1 ml. of enzyme solution containing 15 gamma of protein was added to a reaction mixture (in the Beckman

cell) consisting of 2.5 ml. of the cysteine-pyrophosphate buffer and 0.2 ml. of DPN (final concentration = 1.27×10^{-7} moles per ml.) at 25°. In this mixture at this dilution the enzyme is stable for at least 30 minutes. The reaction was started by the addition of 0.2 ml. of a fresh 1:1 mixture of 5.3 per cent sodium arsenate and triose phosphate² (final concentration = 1.27×10^{-7} moles per ml.). Bimolecular rate constants were calculated from the equation:

$$K = \frac{1}{t} \frac{x}{a(a-x)}$$

where a is the initial concentration of triose phosphate and DPN, and x is the amount of reduced DPN formed in time t (minutes). K was found to decrease with time, but the K values based on 1 minute readings were proportional to enzyme concentration.

In testing for inhibition of the enzyme by antiserum, up to 0.3 ml. of serum could be substituted for part of the cysteine-pyrophosphate buffer in the reaction mixture without producing a pH change that in itself would affect enzyme activity. Unless otherwise indicated antiserum was added to the reaction mixture containing buffer, enzyme, and DPN and given 10 minutes' incubation at 25° before starting the reaction. The per cent inhibition of enzyme activity was calculated from the 1 minute K values determined from rates with and without serum in the reaction mixture.

Inhibition of Yeast d-Glyceraldehyde 3-Phosphate Dehydrogenase by Antiserum.

—When rabbit antiserum was tested in the system described, it was found that the antigen-antibody combination, as measured by the extent of inhibition, was complete within the 10 minute period allowed. The turbidity developing in the cell due to antibody-enzyme flocculation also reached a maximum during this period. The absorption at 340 μ due to the presence of a precipitate was small and was corrected for in the initial reading.

With increasing amounts of antiserum the degree of inhibition increased almost linearly up to about 80 per cent as shown in curve B of Fig. 1. Beyond this the curve levels off never reaching complete inhibition.

The percentage inhibition of 15 gamma of crystalline enzyme given by 0.05 ml. of antiserum was used as a measure of antibody titre. With nearly all antisera tested the inhibition produced by this amount of serum fell on the linear part of the curve. There was a fair correlation between the precipitin titre and the degree of inhibition by the antisera (Table I).

The residual activity with antibody excess as shown in curve B, Fig. 1, suggested that the insoluble enzyme-antibody complex itself retained activity amounting to about 10 per cent of the original enzyme activity. This was confirmed by finding that removal of the precipitate by centrifugation left a supernatant fluid with no enzyme activity, while the washed precipitate showed an activity only slightly lower than the residual activity seen in curve B. Resuspended precipitate settled rapidly and somewhat higher rates were obtained when the reaction mixture was stirred between readings.

² We are indebted to Dr. H. O. L. Fischer, Dr. E. Baer, and Dr. H. A. Lardy for a sample of synthetic dL-glyceraldehyde 3-phosphate (11) supplied to this department.

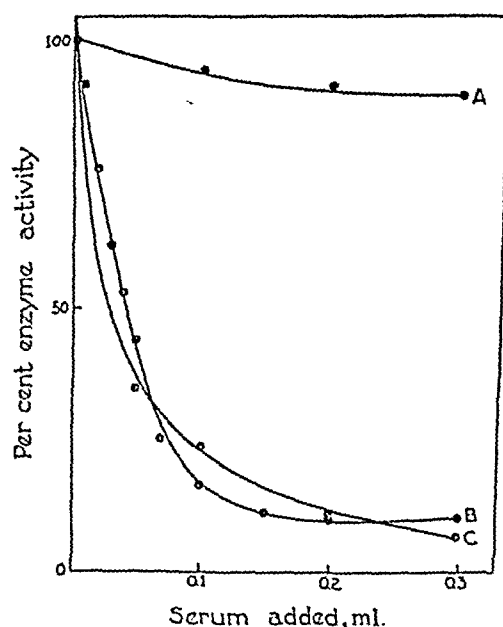


FIG. 1. Effect of increasing amounts of rabbit and chicken antisera on the activity of 15 gamma of yeast *d*-glyceraldehyde 3-phosphate dehydrogenase. Curve A shows the slight degree of inhibition caused by normal rabbit serum. The inhibition by normal chicken serum is of the same order. Curve B shows the effect of rabbit immune serum with a precipitin titre of 1:10,000. Curve C is with chicken immune serum of the same precipitin titre.

TABLE I
Inhibition of Yeast Enzyme by Antiserum

The degree of inhibition of 15 gamma of yeast *d*-glyceraldehyde 3-phosphate dehydrogenase by 0.05 ml. of different rabbit antisera is compared to the precipitin titre of the sera.

Serum	Inhibition	Precipitin titre, highest antigen dilution
	per cent	
A (5)*	20	1:100
B (5)	25	1:1,000
C (10)	32	1:1,000
D (20)	79	1:10,000

* The numbers in parentheses indicate the number of injections of antigen given to each rabbit.

Constant degrees of inhibition of the yeast enzyme by chicken antisera were obtained when enzyme, DPN, and antiserum were incubated before buffer was added to the reaction mixture. Under these conditions, in which the amount of serum dilution is small, the reaction of enzyme and antibody is rapid, and

the extent of inhibition and precipitation is maximal within a few minutes. Curve C of Fig. 1 shows the effect of increasing amounts of chicken antiserum on the yeast enzyme.

Relationship between Enzyme-Antibody Combination, DPN, and Substrate.—An excess of rabbit antiserum (2 times the amount necessary to give maximal inhibition of the enzyme) was incubated with the yeast enzyme in cysteine-pyrophosphate buffer. The residual activity of this incubation mixture was determined at three concentrations of DPN and triose phosphate, 1.25, 2.54, and 5.08×10^{-7} moles per ml. for each component. In each case the activity found was 5 per cent of the activity of enzyme alone determined at these concentrations. The activity measured in these experiments was that of the enzyme-antibody precipitate, which gave no evidence of dissociation at increasing concentrations of DPN and triose phosphate.

DPN was found to have an effect on the degree of inhibition of the enzyme by antiserum, if it was present *during* the combination of enzyme and antibody. This was most easily seen with small amounts of antiserum. In a typical experiment 0.05 ml. of rabbit antiserum was added to a reaction mixture already containing DPN, enzyme, and cysteine-pyrophosphate buffer. The activity determined after 5 minutes' incubation by addition of arsenate-triose phosphate showed 31 per cent inhibition of the enzyme. Longer incubation did not increase the extent of inhibition. When DPN was absent during the preliminary incubation of antiserum and enzyme, 54 per cent inhibition of activity was found. The yeast enzyme itself was stable without DPN. Precipitates obtained with antibody excess showed somewhat higher activities when enzyme and antibody combined in the presence of DPN. Chicken antisera behaved similarly to the rabbit antisera in respect to DPN. As far as could be determined, triose phosphate had no influence on enzyme-antibody combination.

Rate of Combination of Enzyme with Antibody.—The combination of antibody and enzyme was studied by addition of antiserum to enzyme, while the enzyme was acting on its substrates. High concentrations of triose phosphate and DPN were used, and under these conditions the rate was nearly linear after the first half-minute as shown in curve A of Fig. 2. Rabbit antiserum was added to identical reaction mixtures at the 3rd minute and the rate followed until it again became linear, indicating completion of enzyme-antibody combination. In curve B the amount of antiserum added was slightly less than that necessary to inhibit the enzyme maximally. Curves C and D were with 3 and 6 times the amount added in (B), respectively. Tangents to the curves have been drawn to show the points at which the slopes are approximately half the difference between the control rate (A) and the residual rate shown in (D). These points indicate the time at which the enzyme is half maximally inhibited and presumably half combined with antibody. In (B)

this point is 2 minutes after addition of antiserum, in (C) about 1 minute, and in (D) less than $\frac{1}{2}$ minute.

In other experiments enzyme and antibody were allowed to react for varying times in the absence of either DPN, triose phosphate, or both. The reaction

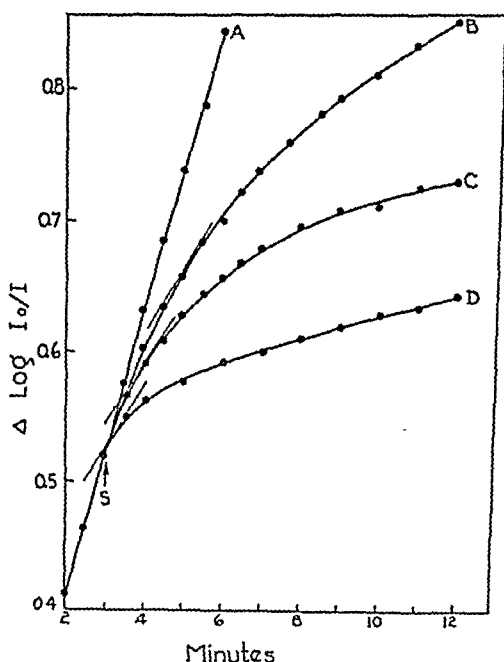


FIG. 2. The addition of rabbit antiserum to the enzyme, while it is acting on its substrate. Curve A shows the rate of the uninhibited reaction with 1 gamma of the yeast dehydrogenase per ml., when the initial DPN and triose phosphate concentration are 1×10^{-6} moles per ml. for each component. Curves B, C, and D show the effect of three amounts of antiserum (S) added at the 3rd minute (arrow) to identical reaction mixtures. All reactions are started with the addition of the triose phosphate. For further description see text.

was started by the addition of the missing component or both components simultaneously. The rate became linear in every case after the same total time intervals of antigen-antibody combination as in the experiments in which antiserum was added during the reaction.

Specificity.—Rabbit antisera to the yeast *d*-glyceraldehyde 3-phosphate dehydrogenase were tested for possible inhibitory effect on hexokinase isolated from the same strain of bakers' yeast. No inhibition was noted, antisera and normal rabbit serum both having a slight activating effect presumably due to protective protein action on the hexokinase (9). These results are in agreement with the findings of TenBroeck (12) and McCarty (13), who found no evidence of a serological relationship between different enzymes from the same organ of the same species.

Four rabbit and two chicken antisera to the yeast enzyme were tested for cross-reaction with the analogous enzyme from rabbit muscle. Muscle *d*-glyceraldehyde 3-phosphate dehydrogenase was substituted for the yeast enzyme in the test system previously described. When the enzyme was incubated

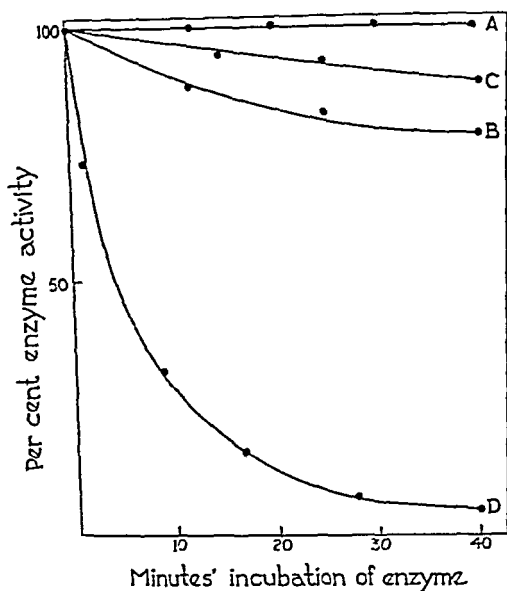


FIG. 3. The effect of one atypical rabbit antiserum to the yeast enzyme on the activity of the muscle dehydrogenase. 10 gamma of the crystalline muscle enzyme was used in each case. Curve A shows the stability of the enzyme when incubated in buffer with DPN. Curve B shows the slow downward drift of the enzyme under the same conditions without DPN. Curves C and D show the effect of 0.1 ml. of the antiserum on enzyme activity during incubation with and without DPN, respectively. The final concentrations of DPN and triose phosphate were 1.27×10^{-7} moles per ml. for each component.

with antiserum in the presence of DPN, only the same slight degree of inhibition as that given by normal sera was noted. When the incubation was carried out in the absence of DPN, lower activities were found, but with only one antiserum was the effect definitely greater than that seen with enzyme alone or with enzyme and normal serum. (This enzyme is less stable than the yeast enzyme in the absence of DPN).

The one antiserum found to inactivate the muscle dehydrogenase was from one of two rabbits that had received four courses of injection of the yeast enzyme. With this particular serum the effect on the muscle enzyme is shown in curve D of Fig. 3. Curve C shows the protective action of DPN on the enzyme incubated with the same serum. Curves A and B show the relative stability of the enzyme incubated in buffer alone with and without DPN. With

eight normal rabbit sera tested and the other immune sera, the enzyme showed the same degree of stability without DPN as in curve B.

When a sample of this serum was completely absorbed with the yeast enzyme (homologous antigen) until no further precipitation occurred, followed by removal of the precipitate by centrifugation, it showed no decrease in its inhibitory action on the muscle enzyme. This result and the failure to detect any inhibition by other antisera make it doubtful that the inactivation of the muscle enzyme by this antiserum should be called an immune reaction.

DISCUSSION

Some enzymes have been found to be 70 to 95 per cent active when combined with specific antibody, even when precipitation of the enzyme occurs (14-17). Other enzymes (1, 2, 13, 18) have been shown to be strongly inhibited by their immune sera. In the latter group with two of the enzymes studied, there is evidence that one point of antigen-antibody combination may be at the active center of the enzyme. Yeast *d*-glyceraldehyde 3-phosphate dehydrogenase is 90 to 95 per cent inhibited by antiserum, and in this case the combining site of the coenzyme, DPN, appears to be involved.

The effect of DPN on the inhibition of the yeast dehydrogenase is different from the retarding effect of lecithin on the lecithinase-antilecithinase reaction studied by Zamecnik and Lipmann (2). DPN appears not to slow the reaction of antigen and antibody, but its presence results either in less antibody combining with the enzyme or in the formation of a precipitate having a higher activity.

SUMMARY

Antibodies to yeast *d*-glyceraldehyde 3-phosphate dehydrogenase have been produced in rabbits and chickens. The antiserum from either animal inhibits the activity of the yeast enzyme with the formation of a precipitate that has a low residual activity. One combining site of antibody on the enzyme appears to be the point of DPN attachment.

The rate of antigen-antibody formation has been studied with varying concentrations of antibody.

The activity of yeast hexokinase is not inhibited by the antiserum to the dehydrogenase.

Five of six antisera to the yeast enzyme showed no inhibition of the analogous enzyme from rabbit muscle, but one serum strongly inhibited the muscle dehydrogenase in the absence of DPN. No evidence was obtained that the inhibition by this one antiserum was due to an immune reaction, because the inhibition persisted after absorption with the homologous antigen.

The authors wish to thank Dr. Carl F. Cori for his interest and guidance in this work.

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HEREDITARY OSTEOPETROSIS OF THE RABBIT

I. GENERAL FEATURES AND COURSE OF DISEASE; GENETIC ASPECTS

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PLATES 33 TO 35

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From our increased knowledge of the natural history and biology of the rabbit largely attained in recent years, it appears that this species is subject to a much greater number of inherited morbid conditions than had heretofore been generally thought. The conditions vary widely, ranging from those which are comparatively simple and apparently non-injurious to those with more or less severe manifestations and even lethal effects. In the first category are found such anatomical and physiological variations as the Rex type of coat, yellow fat, and brachydactylia and allied abnormalities (1). In extreme contrast to the essentially harmless conditions which are in nowise incompatible with growth and development are those associated with non-viability at or within a few hours of birth. Some of these so called lethals are ill defined and little of a precise nature is known about them but in the case of achondroplasia, for example, many features have been fully described (2-4).

Hereditary osteopetrosis, which is reported in this and succeeding papers (5, 6), is likewise present at birth but the uniformly lethal effect is delayed, usually to the 4th or 5th week. It is one of a number of disease complexes which have been encountered in a colony of rabbits maintained in this laboratory for experimental investigation on constitutional factors associated with susceptibility and resistance to disease.

Marble bone disease of man was first described in 1904 by Albers-Schönberg (8). The name osteopetrosis, which was introduced by Karshner (9) in 1926, is now commonly employed although other descriptive names such as osteosclerosis are sometimes used. The disease was long considered a rare affection, but with the great increase of x-ray examination, its incidence now appears to be not infrequent. Its outstanding feature is the remarkable appearance and structure of the bones, the chief characteristics of which are an extremely thick cortex and a narrowed marrow cavity which is filled to a greater or less extent with abnormal medullary bone. In severe cases, there is an accompanying anemia which is often pronounced, and various symptoms such as physical underdevelopment, tooth defects, spontaneous fractures, hydrocephalus, and optic atrophy are described.

* Dr. Brown died on August 4, 1942. The disease here described occurred in stock with which he was working and the early phases of this study were carried out by him.—L.P.

The disease is most frequently seen in infants and young children and it now seems clear that its onset takes place at some phase of intrauterine development (10). Its etiology is unknown. A significant feature is its strong familial tendency, and its hereditary character is generally accepted. In the opinion of most observers, the mode of inheritance is on the basis of an autosomal recessive factor, but the occurrence of certain parent-offspring cases raises the question of an irregular dominant factor (11-13).

The hereditary disease of the rabbit here described for the first time closely resembles in many respects osteopetrosis of man. Investigations on the human disease have been greatly limited by the relatively small number of cases on which comprehensive studies were possible. The present comparable condition of the rabbit should be of value, particularly from the standpoint of an experimental approach to the human problem. Furthermore, since embryological material is now available, it seems not unlikely that investigations in this field might have important applications in the basic problems of osteogenesis and hematogenesis.

The observations on hereditary osteopetrosis of the rabbit have been divided into three categories for presentation. The present paper is concerned with the manifestations and course of the disease and the results of genetic studies. The following paper (5) contains a description of x-ray examinations of the skeleton and the results of hematologic observations and of chemical determinations. In a third paper (6) the results of postmortem examinations and histological studies will be described. A summary of these studies has recently been reported (7).

Materials and Methods .

The foundation of the osteopetrosis stock was a small group of pure bred Dutch rabbits purchased from a dealer, which included in particular a male rabbit some of whose offspring from backcross matings were the first observed examples of the condition. As soon as the hereditary nature of the condition appeared probable, extensive interbreeding of a large number of more or less closely related animals was undertaken for the purpose of obtaining additional transmitters. The identification of transmitters was made on the basis of affected progeny and several were discovered in these early experiments. Enlargement of the stock was accomplished by using identified transmitters for further inbreeding with related animals and also for outcrosses with various unrelated stocks. Progeny derived from such outcross matings constituted an F_1 hybrid generation which was used as the basis for genetic studies.

The present report is based upon 293 rabbits which at birth or within a few days showed the characteristic physical signs of the disease complex. These rabbits were contained in 169 litters. The control material comprised the 722 normal litter mates and other normal rabbits of comparable ages derived from both related and unrelated stock.

A detailed description of affected individuals was included in the notes dictated at the time the litter was first examined a few hours after birth. The animals were weighed on an automatic balance calibrated in gram intervals and their sex recorded. Subsequent examinations were made 3 times a week or oftener, particular attention being paid to the growth and nutri-

tional state of the abnormal as compared with the normal members of the litter. Body weight determinations were usually a reliable index of the general condition of the animal. In the great majority of cases, the diagnosis was confirmed by x-ray photographs and post-mortem examination of the skeleton.

All adult rabbits in the colony are housed in large individual cages, and the diet comprises a constant water supply, timothy and alfalfa hay, and a standard pellet preparation of the best quality obtainable. Supplementary feeding of fresh cabbage and carrots is carried out during the winter months. An excellent condition of health is generally maintained. In breeding experiments, the duration of pregnancy can be calculated to within a few hours. The time of most matings is exactly known and in those cases in which the doe is left with the male, it is known to within 18 hours. Pregnancy checks are made 10 days after mating and pregnant females are examined at regular intervals throughout gestation. The detection of abnormal or unusual features in this period is thus reasonably assured.

RESULTS

Osteopetrosis of the rabbit has as characteristic features abnormal bone and tooth development, retardation of growth, anemia, progressive cachexia, and an invariably fatal effect. The dense bone shadows with reduced marrow spaces, particularly of the long bones, are striking features of x-ray photographs (5).

Of 293 animals showing the disease, 275 were born alive while 18 were found dead when the litter was first examined a few hours after birth. Almost all litters were born at term and there was only an occasional instance of prematurity or of slightly prolonged gestation. No abnormal or unusual conditions were observed during pregnancy which could be associated with the disease. The affected animals were the only members of the litter which at birth or within the first few days of life showed abnormalities that could be attributed to the disease. Transmitters could be identified only by breeding tests.

Many of the cases were among the smaller individuals of the litter at birth. The great majority were well nourished and continued to grow for some days. The fur and nails were well developed and the nares were patent. The characteristic tooth abnormality observed during life comprised the subnormal development or the absence of one or more of the incisor teeth. The disease is uniformly fatal; the survival period of most cases was 4 to 5 weeks.

There were 17, or 5.8 per cent, of the rabbits classified as tarda cases because of the delayed development of incisor tooth abnormalities. The teeth appeared to be normal at birth but within 1 to 10 days characteristic changes occurred. The animals were generally large individuals and several lived somewhat longer than the usual case.

The signs and course of the disease will now be described, after which the results of genetic studies are presented. It will be helpful in reading this description to refer to the x-ray photographs in an accompanying paper (5); these illustrations have been arranged in order of disease progression.

General Description

Teeth.—The first clue to the diagnosis of osteopetrosis is an abnormal condition of the incisor teeth which is usually present at birth. The molar teeth are similarly affected as postmortem examination has regularly revealed; but the posterior portions of the buccal cavity are not easily examined during life.

In the normal rabbit born at term, the four incisors are fully erupted and the teeth of each pair are evenly placed in line with and in close approximation to each other. The enamel is smooth, glistening, and white, usually with a faintly bluish tint and slightly translucent.

The chief attributes of the incisor tooth abnormality were an initial subnormal development and subsequent retarded and abnormal growth. The most frequently observed condition at birth was the absence of, or the rudimentary state of one or more of the teeth. In a good many cases, the point of an unerupted tooth was palpable through the gum but in others nothing of this sort could be felt. The absence of one or both uppers occurred more frequently than a similar condition of the lower incisors while the absence of all four teeth was occasionally observed. In some cases, only one incisor was seen. The appearance of the incisor teeth on the 2nd day of life of a normal rabbit and a litter mate with osteopetrosis is illustrated in Fig. 1; in this case the upper incisors were not erupted and the lowers were slanting and convergent.

There were also at birth less obvious tooth abnormalities comprising some peculiarity of size, shape, color, or position. In many of these instances, the tooth was somewhat small, and was apt to be out of line with the other member of the pair and more or less separated from it, the cutting edge was frequently curved or slanting or somewhat pointed instead of straight, and the enamel had an opaque slightly yellowish color. Various combinations of incisor abnormalities were seen, as for example, an absent pair and one abnormal and one normal appearing tooth of the other pair. Occasionally only one of the four teeth was suspiciously small.

Within a few days, the condition noted at birth became accentuated and other abnormalities were apt to occur as well, as can be seen in the photographs of several cases from 6 to 35 days of age (Figs. 2 to 7).

The abnormalities most frequently observed as the disease progressed were a retardation or suppression of growth of the teeth and shedding of them.

Small stunted teeth were apt to be deformed and abnormally placed and often the pair was convergent (Fig. 2) or more or less widely separated (Fig. 5). The enamel of these abnormal teeth was often an opaque dirty grey color or a dull yellowish white. In some cases the incisor teeth retained their infantile proportions with no apparent growth whatever. Such teeth were usually widely separated and rotated or twisted in position. The photograph in Fig. 6 illustrates this condition of tiny peg-like teeth in a 21 day old case. On the other hand, there were a few instances in which an originally small tooth became a thin spindly fragile structure which might be longer than normal (Fig. 7).

The shedding of an abnormal tooth or even of an apparently normal one was often observed (Figs. 2, 3, 4, and 7). It usually occurred in the first few days of life but sometimes not until the animal was a week or 10 days old. The absence of all four incisors in a 9 day old rabbit is shown in Fig. 3. In this case at birth the left upper incisor was missing, the right upper was a small short tooth, and the lowers appeared to be normal; the three teeth were shed between the 6th and 9th days. An x-ray photograph of this rabbit is reproduced in the following paper (5, Fig. 5).

In another case of a 13 day old rabbit shown in Fig. 4, a small right lower incisor was shed between the 4th and 6th days. Both upper incisors were missing from birth. X-ray photographs of the jaws and other bones of this animal also appear in the following paper (5, Fig. 9).

The eruption of a second tooth in the gum area of the shed incisor was a fairly common occurrence; usually the new tooth was rudimentary and grew but little (Fig. 5). There also were instances in which an incisor erupted, usually within the first 2 or 3 days of life, in an area in which at birth no tooth was seen or felt. Such teeth were practically always stunted and deformed and remained in this condition.

As has already been mentioned, certain animals have been classified in a group designated as osteopetrosis tarda on the basis of a delayed occurrence of incisor teeth abnormalities. At birth the teeth appeared to be normal but typical changes soon developed and in the majority of animals at 3 to 4 days of age. The subsequent general course of events was comparable to that of the usual case.

Shedding of incisor teeth was relatively frequent in tarda cases and was often followed by the abnormal growth of one or more of the remaining teeth as is illustrated by the photograph of a rabbit 35 days of age (Fig. 7). At this time the right upper incisor was a short stumpy tooth which slanted toward the midline and was widely separated from the smaller somewhat pointed left upper incisor. Both lower incisors were abnormally long and widely separated. The enamel of all four teeth was dull, opaque, and white. At birth, the teeth appeared normal but at 3 days of age little or no development of the uppers was apparent and by the 10th day the subnormal condition was marked. By the 16th day, the left upper was missing and the lowers were longer than normal. On the 25th day, there were two very short stumpy upper incisors, a second left upper meanwhile having erupted. In the next 10 days, there was slight growth of the uppers but a considerable overgrowth of the lowers. During this period the general condition of the animal underwent a rapidly progressive deterioration.

It has been mentioned that abnormalities of the molar teeth similar to those of the incisors were observed at postmortem examination. Many of these teeth were not erupted while others were small and irregularly placed. It was evident that the underlying condition responsible for the abnormal condition of the incisor teeth was not confined to the anterior regions of the jaws but that it extended throughout both bones. Cleft palate was not seen.

Size.—At birth, rabbits with osteopetrosis tended to be slightly smaller than their normal litter mates. On the basis of mean body weights the difference was comparatively minor. In a group of 161 cases and 353 normal sibs, for example, the mean birth weights were 44.5 and 46.2 gm. respectively. The frequency distribution curves of birth weight values, however, give a better idea of the size variations encountered.

The curves in Chart 1 were drawn at 5 gm. intervals. The one representing the birth weights of normal rabbits approaches a normal frequency curve and its peak practically coincides with the mean value for the group. The curve representing the diseased rabbits is more irregular and does not reach as high a level. Furthermore, the position of its two highest

points is to the left and below the peak of the normal curve. One of these two points representing the 35-39.9 gm. class is only slightly lower than that of the next highest class in which the mean value of 44.5 gm. falls. These classes comprise 60 animals or 37.3 per cent of the group; with the inclusion of the next highest class, 45-49.9 gm., a total of 86 cases, or 53.4 per cent of the group, is represented. A similar comparison with the normal sibs shows that in the 45-49.9 gm. class which contains the mean value, there were 79 or 22.4 per cent of the total group represented. With the inclusion of the two adjacent classes of 40-44.9 and 50-54.9 gm., the number of animals represented is 183 or 51.8 per cent of the group. Slightly more than half the rabbits of each group fall in the class containing the mean weight value and

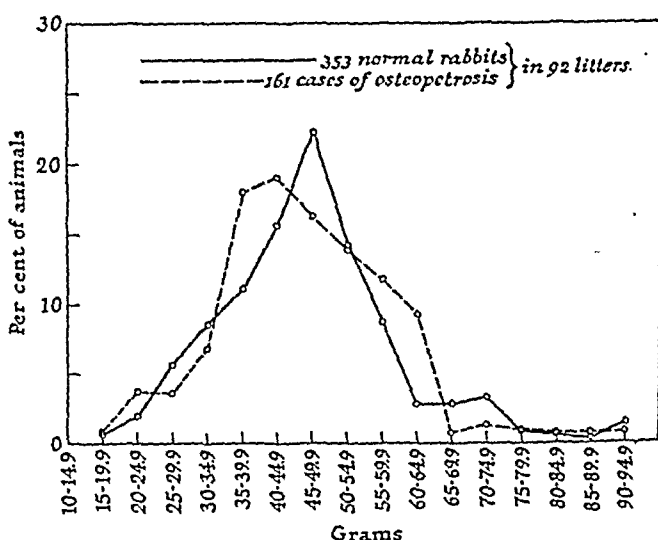


CHART 1. Distribution frequencies of birth weight values.

the two adjacent weight classes, but the position of these classes for the diseased rabbits is to the left and slightly below that for the normal litter mates.

A comparison has also been made of the relationship of the weight of each individual in a litter expressed in terms of a percentage of the weight of the heaviest litter mate (14). This procedure eliminates the variations due to differences in the absolute weights of animals of different litters since all observations are given a relative value. The percentage values were grouped in classes in descending order of magnitude while the frequencies in each class were expressed in relative or percentage values of the total number of rabbits. The results of this analysis are shown in Chart 2. The curve representing the 161 cases of osteopetrosis generally resembles that for 353 normal litter mates but its comparative height and position indicate the tendency of these cases toward small rather than large size.

Course of Disease and Length of Survival.—As has already been pointed out, there is nothing unusual or peculiar in the general appearance of osteopetrosis animals at birth or for the first few days of life except the abnormal condition

of the teeth. The animals are well developed and well nourished. Nursing usually proceeds normally, there are no obvious gastrointestinal disturbances,

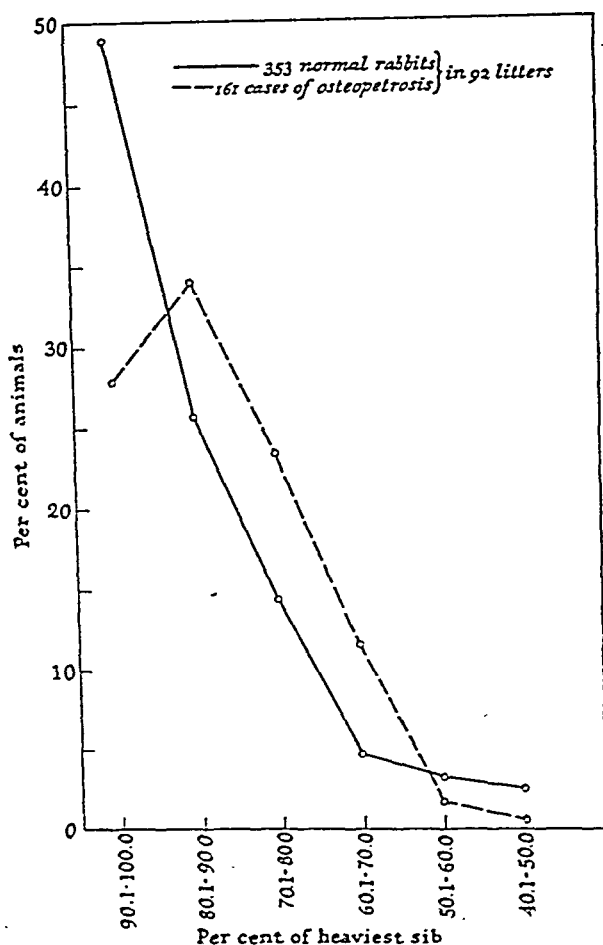


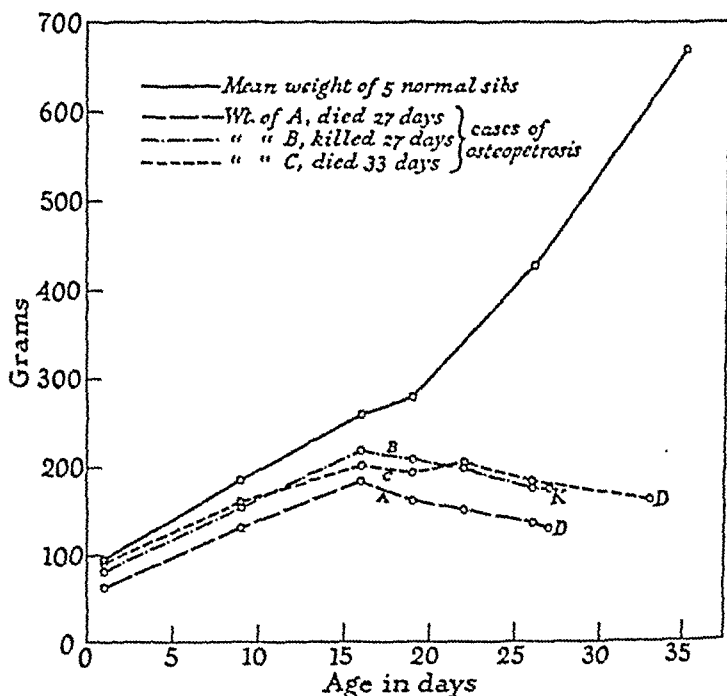
CHART 2. Distribution frequencies of birth weight values in terms of the per cent of the heaviest sib. The number of animals in each weight class is expressed as a per cent of the total number of animals.

the body weight increases, and growth is evident. Within a short time, however, often by the 14th day or even earlier, signs occur, the most striking being a retardation of growth followed by the development of an outspoken cachexia. The rate of body weight gain decreases and ultimately there is actual weight loss. These several features are illustrated by the weight curves in Charts 3 and 4.

It will be noted from the curves in Chart 3 representing 3 cases of oste-

opetrosis, that all body weight values were smaller than the mean values of 5 normal sibs.

All the diseased rabbits, A, B, and C, continued to gain weight for a fortnight at approximately the same rate as the normal animals. From this time on there was a progressive weight loss with the exception of one temporary reversal in the case of rabbit C from the 19th



• CHART 3. Body weight values of 3 rabbits with osteopetrosis and 5 normal litter mate rabbits from birth to 33 days of age. For the normal sibs, mean values are used.

to the 23rd day. The first death, that of rabbit A, occurred on the 27th day; the body weight had decreased to the level reached on the 9th day. Rabbit B, killed on the 27th day, was in quite good physical condition with a weight only slightly lower than that on the 16th day. Rabbit C survived to the 33rd day; the body weight was then at a somewhat lower level than the peak value on the 16th day. Meanwhile, all 5 normal sibs continued to grow rapidly, particularly after the first fortnight. At the time rabbit C died the mean weight value of the normals was approximately 4 times the weight of C.

Chart 4 contains the curves representing body weight values of two normal and two osteopetrosis litter mates.

At birth and at 3 days of age, the weights of all the animals were practically identical but from then on, both normal animals, A and B, gained weight much more rapidly than C and D, the rabbits with osteopetrosis. In the case of rabbit D there was a stabilization of weight on the 9th and 11th days and a slight loss on the 13th day when the animal was killed. In the

case of rabbit C, a similar weight stabilization occurred but on the 13th day a slight gain was found and further gains took place until the 23rd day; thereafter there was a loss of weight and the animal was killed on the 27th day. At this time the weight of one normal was $2\frac{1}{2}$ and that of the other normal was 3 times the weight of rabbit C.

There was a considerable variation in the course of the disease particularly with respect to the time of development of the cachexia and its rate of progress. The opening of the eyelids usually took place at the normal age of 10 to 11 days

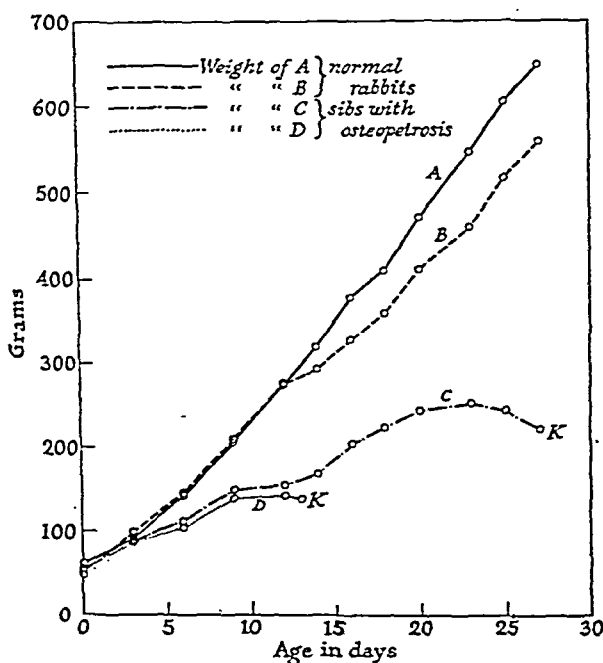


CHART 4. Body weight values of 2 rabbits with osteopetrosis and 2 normal litter mate rabbits from birth to 27 days of age.

but a delay of 2 or 3 days was not infrequent and in these cases the gain in weight was often comparatively slight and death was apt to occur early, that is, in the 2nd and 3rd weeks. In the majority of cases, however, the course of events was more prolonged, the animals continued to nurse, and many exhibited a surprising activity in spite of evident malnutrition and progressive weight loss. The small size of affected rabbits particularly after the 2nd week was very striking.

An idea of the manifestations and course of the disease can be obtained by comparing the photographs of three cases with those of three normal litter mates. The appearance of the first case and a normal sib on the 7th, 15th, and 27th days is shown in Figs. 8, 9, and 10, respectively. The photographs of

another pair on the 16th and 24th days appear in Figs. 11 and 12, while a third example on the 34th day is shown in Fig. 13.

The most obvious feature of these photographs is a difference in size, the retardation of growth becoming more striking with increasing age and disease progression. It was already apparent at 7 days of age in the case pictured in Fig. 8 and it was marked at the older ages illustrated in Figs. 9 to 13. As the disease progressed other signs developed. The fur became dull and somewhat rough or irregular and fluffy or nappy (Figs. 10 and 12). Eventually, a spotty thinning of the coat might occur and sometimes there were small areas of alopecia particularly in the cervical region. An ophthalmia was frequent. The eyelids were apt to droop, the corneas appeared dull, lacrimation was likely to be excessive, and often tiny crusts or scales accumulated on the lids (Figs. 10 to 12). Not infrequently the secretion was thick and gummy and occasionally the lids were sealed.

By the 4th week or even sooner, emaciation and evident weakness were usually present. In these cases the skin lost its normal tautness and the musculature felt soft and flabby. As the disease progressed, malnutrition and weakness became increasingly pronounced with eventual prostration and death. A few days before death, a thin mucous or watery diarrhea often developed. In some cases, the abdomen became prominent and bloated. The rabbit whose photograph on the 16th and 24th days is reproduced in Figs. 11 and 12 showed a steadily deteriorating condition and was killed, together with the normal litter mate, on the 27th day. The x-ray photographs of the skeletons appear in the following paper (5, Figs. 17 and 19).

The great majority of deaths occurred in the 4th and 5th weeks as is shown by the frequency distribution curve of the length of survival of 243 cases (Chart 5). It should be mentioned that 34 of these animals, or 14 per cent, were killed for blood chemistry determinations and other studies. Their distribution is indicated on the chart and it will be noted that in most age groups, their number in proportion to the total number of animals in the group is small. There were two exceptions. In the 13-15 and the 19-21 day groups, the proportion of killed animals was 53.3 and 38.9 per cent respectively. From what is known of the disease, it is probable that the older cases at least would not have survived much longer. However, it should be pointed out that contrary to the usual experience with diseased rabbit nurslings, predictions of probable length of life, especially of cases surviving more than a fortnight, were not too reliable. Some rabbits whose general condition did not seem immediately critical promptly died while others continued to live for several days in spite of marked emaciation.

As has already been pointed out, certain of the longer lived cases belonged in the tardy class of the disease. There were 17 such animals in the total group of 243 cases represented in Chart 5; their distribution is shown by vertical bars at the base of the chart. It will be noted

that 13 lived longer than 2 weeks and that 8 of these died in the 5th, 1 in the 6th, and 1 in the 10th week of life respectively.

Hydrocephalus.—Other signs of the disease which have been seen more or less frequently will now be described beginning with hydrocephalus. At first this condition was thought to be fairly common and together with the manifestation of prominent eyeballs, was responsible for the laboratory nickname of "hydro-bugeye." Although some instances of hydrocephalus were found at birth most of them developed within the 1st week of life. Further experience, however,

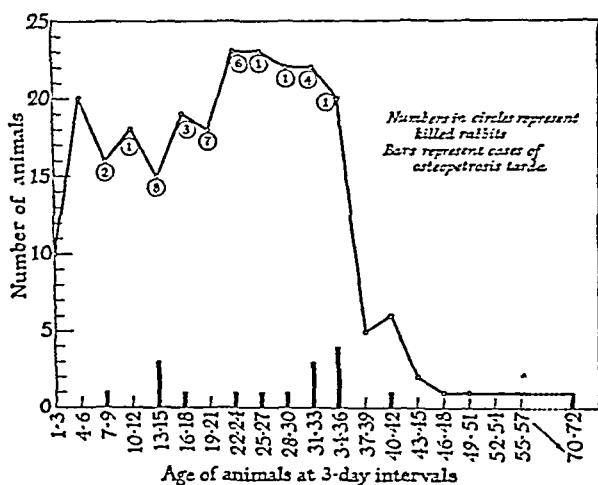


CHART 5. Distribution frequencies of length of survival of 243 rabbits with osteopetrosis, including 34 that were killed.

did not support the early impression of the comparative frequency of hydrocephalus and, actually, its incidence rate is not known. Postmortem examination of many cases in which a slight or a fair degree of hydrocephalus was thought to be present failed to reveal any evidence of it. The prominent calvarium and apparently increased fluctuation over the posterior fontanelle were deceptive. The history of similar cases together with the character of the skull bones at different ages suggests that the probable explanation lies in the deficient calcification of the skull and delayed closure of the sutures which give the impression of floating bones. The pressure of the growing brain on pathological bones may also be a contributing factor.

On the other hand there were definite instances of hydrocephalus in which the diagnosis made during life was confirmed at autopsy. In most of these unmistakable cases, a progressive dome-like expansion of the posterior calvarium occurred over a period of a few days. Some of these cases were held for observation and in a few of them gradual regression of the condition took

place and only a minor degree of hydrocephalus was found at postmortem examination. Finally there were still other cases in which hydrocephalus was not suspected during life but which showed this condition at autopsy (6).

Exophthalmos.—As has just been mentioned, prominence of the eyeballs was observed and in a few cases extreme grades amounting to well marked protuberance developed. Certain conspicuous examples are known to have occurred in hydrocephalic cases, but in others scarcely less pronounced, no evidence of hydrocephalus was found at autopsy. Although the condition was not marked in the majority of cases, the incidence of some slight or minor degree of eyeball prominence was fairly frequent. It was noted at birth or within a day or so and usually it had subsided after about a week or 10 days. It appears likely that the circumstances just suggested as being responsible for the erroneous diagnosis of hydrocephalus were also directly related to the manifestation of prominent eyeballs.

Neurologic Signs.—There were a few cases in which uncoordinated and athetoid movements of the head and body were seen. These symptoms usually developed within the first 3 or 4 days but sometimes they were not noted until the 2nd week. Tremors of the head and occasionally of the entire body have also been observed. In certain animals there was a lateral spasmodic shaking of the head toward one side only or sometimes toward one side and then toward the other. Other animals displayed a peculiar weaving motion of the head. There were a few instances of opisthotonos and in one case, it was marked. Jerking movements of the eyeballs, a nystagmus, have occurred. In some rabbits, the movements suggested an interference with vision and in addition, the face had a strange blank expression. In these cases the pupillary light reflex was thought to be sluggish or absent.

The majority of the cases were examples of an early fatal outcome but a few survived for upwards of 3 weeks. In the latter individuals particularly it was noted that there was a considerable variation with respect to the constancy of the peculiar movements. At one examination, for example, the condition was striking, but on the next it might be comparatively minor or practically absent. It should also be mentioned that the development of the neurologic signs was not necessarily associated with malnutrition or advanced cachexia.

With the development of unusual disturbances of movement and possibly of vision, the presence of hydrocephalus was suspected and searched for. Again, it was demonstrated in some animals but not in others.

Genetic Data

As has already been stated, the individuals known to represent cases of osteopetrosis were the only members of their litters which showed any abnormalities at birth or subsequently which could be directly attributed to the disease. X-ray photographs of a large number of these apparently normal rabbits were taken not only for control purposes in connection with their osteopetrosis litter mates but also with the possibility in mind that abnormal bone conditions might be disclosed in some animals. None was found.

Since the disease was invariably lethal within a few weeks and since transmitters could be identified only by breeding tests, genetic studies were necessarily carried out with such animals. A number of identified transmitters were mated with unrelated rabbits, including examples of pure breeds, to form an F_1 hybrid generation, and these animals in turn were tested for transmission of the condition. Those found to be transmitters were then interbred to form an F_2 generation.

The 293 cases of osteopetrosis here considered occurred among 1015 total births contained in 169 litters. The incidence of the condition is 28.87 per cent. On the basis of a simple recessive factor, one-fourth, or 254 animals, would be expected to show the condition. There is no significant deviation between the observed distribution of rabbits with osteopetrosis and normal litter mates and the expected distribution on the basis of genetic principles as shown by the chi-square test of homogeneity ($\chi^2 = 1, P = 0.05+$). Of the total number of progeny, 314 rabbits contained in 51 litters were derived from F_2 matings, their F_1 parents being outcrossed hybrids. The number of cases of osteopetrosis in the groups was 79, an incidence of 25.16 per cent.

The disease is not sex-linked. Of a total of 212 cases whose sex was determined, 109 were males and 103 were females. There was likewise no preponderance of either sex in the normal progeny of the osteopetrosis stock nor in the pure bred Dutch stock in which the condition was first seen.

There was no unusual relationship between the frequency distribution of osteopetrosis cases and the size of the litters. The 169 litters contained from 2 to 11 animals, the average number being 6. A curve drawn to illustrate the frequency distribution of the litters in relation to litter size approaches a normal frequency curve. The same is true of the distribution curve representing the number of cases in the several litter classes or sizes.

In analyzing the proportion of the total number of osteopetrosis rabbits to the total number of births in each litter class it was found that in the smaller litters of 2, 3, and 4 animals, the proportion of diseased rabbits ranged from 50.0 to 39.3 per cent. In the litter classes of 5 and 6 animals, the ratios were 34.4 and 30.3 per cent respectively. In the remaining classes of 7 to 11 animals, the ratios varied from 25.8 to 18.1 per cent. The largest number of total births in any litter class, 203, occurred in the class of 7 animals; of these births, 49, or 24.1 per cent, were cases of osteopetrosis.

DISCUSSION

The hereditary morbid condition of the rabbit here designated as osteopetrosis is considered to be a disease entity on the basis of, first, a well defined complex of manifestations and a distinctive course which have been described in the present paper and, second, characteristic bone changes observed in x-ray photographs and in postmortem material. These latter are discussed in later reports (5, 6). The several manifestations are peculiar to this condition and have not otherwise been observed in a large rabbit stock maintained as a colony in this laboratory nor have they been reported elsewhere so far as is known.

The disease is notable in several respects. In the first place, it is present at birth and is invariably fatal; the majority of cases survive to the 4th or 5th week of age. The diagnosis which is made at or within a few days of birth is based upon the peculiar appearance of the incisor teeth. The underlying condition is in reality one of subnormal and disturbed development of the entire skeleton. In anticipation of the observations described in the following paper (5), it may be stated here that the characteristic appearance of the bones in x-ray photographs of the younger cases is one of practically uniform density or opacity with little or no suggestion of differentiation. In older cases, some degree of differentiation is seen.

Retarded growth is constantly observed. At birth, the size of the animal tends to be somewhat small in comparison with normal litter mates and the difference is indicated by body weight values. With increasing age, growth becomes increasingly and markedly retarded and eventually there is actual weight loss. The smaller size of the bones as compared with those of normal litter mates of the same age is very striking (5). Malnutrition, weakness, gastrointestinal disturbances, and prostration are characteristic manifestations. Hydrocephalus has occurred and variable degrees of prominence of the eyeballs are frequently observed. Peculiar muscular movements have occasionally been seen.

The disease is hereditary and the mode of inheritance is on the basis of a simple recessive unit factor. The appearance of unaffected litter mates is entirely normal and transmitters of the disease (heterozygotes) can be identified only by breeding tests. The condition was first observed in the backcross progeny of a pure bred Dutch rabbit.

The condition bears a striking resemblance to the disease of man first described as marble bones and more recently as osteopetrosis. In the severe form of infants and young children, physical underdevelopment is usually observed and the characteristic dense appearance of the skeleton in x-ray photographs is pathognomonic. Other manifestations such as tooth defects, hydrocephalus, and optic atrophy are often present. An anemia is generally found and this is also a prominent feature of the disease of the rabbit (5). Again in anticipation of a later report (6), the resemblance of the bone lesions to those of human osteopetrosis is striking. In both conditions there is a failure or a deficiency in bone absorption. Finally, the human disease shows a strong familial tendency and many authors have stated that it is hereditary, an autosomal recessive factor being concerned. However, certain parent-offspring cases have occurred. Nussey (13) refers to the records of 7 families with 26 cases in whom the disease was observed in more than 1 generation; in 6 families, 2 generations and in 1 family, 3 generations were affected. These observations raise the question of an irregular dominant factor. This difference in inheritance of the human and the rabbit disease, if it exists, is commonly observed in a comparison of the inheritance of the same abnormality in man and in lower animals.

Human cases in all age periods have been observed but the majority are in infants and children (15). The occurrence of cases in young infants together with the general character of the symptoms, and in particular retardation of growth and dentition, has led to the opinion that the onset of the disease is in fetal life. That this actually occurs has been shown by the demonstration of typical findings in a 6 months fetus (10). In a recent monograph, Dierickx (16) states that the disease is characterized as a recessive hereditary morbid condition, the onset coinciding with the first appearance of ossification, about the 2nd fetal month. It has been suggested by several authors that probably many cases die *in utero* and others soon after birth.

The disease is much more severe in younger than in older patients and a comparatively rapid fatal outcome is frequent. In adult cases, on the other hand, the disease is characteristically benign and the presence of typical bone changes is generally discovered only by chance, as from a dental x-ray examination. It is not known when the bone abnormality of these cases was initiated although the presumption is that it began in early or even in fetal life. As the matter now stands, it appears that the survival of the adult case depends largely on the absence of what have been called secondary characteristics of the disease, whereas the early development of these manifestations causes death in a relatively short time (9). Such symptoms as physical underdevelopment, imperfect dentition, hydrocephalus, and in particular anemia, are in all probability incompatible with life.

No explanation for the development of severe manifestations in one individual and not in another is available. The suggestion has been made that actually there are two, or less probably three, distinct conditions having the feature of characteristic skeletal change in common but with other dissimilar symptoms and in addition, different genetic relationships (13, 17, 18). In the case of the severe infantile disease, the operation of a recessive factor has been invoked and for the benign adult condition, a dominant factor. Up to the present, however, the number of cases adequately investigated from an hereditary standpoint is too small for a final opinion on the subject.

The condition of hereditary osteopetrosis in the rabbit is considered to be the counterpart of the severe infantile form of the human disease and the question of a benign adult form with characteristic bone changes as the chief or only abnormality, has not arisen. At least, in an extensive survey of adult rabbits of the stock, including a large number of x-ray examinations, no example of adult marble bones has been found.

Finally, mention should be made of a skeletal condition resembling osteopetrosis which occurs in two other species, the mouse and the Florida manatee, and also in the dugong, which is closely related to the manatee. It is of interest that these species represent such distinctive orders as the Rodentia and Sirenia among the mammals.

In the grey-lethal mouse described by Grüneberg (19-22) the formation of all the yellow pigment of the coat is suppressed, secondary bone absorption is completely absent, the teeth fail to erupt because of the resistance of the bone, growth is retarded, and death occurs between the 22nd and the 30th day of age. Anemia, however, does not develop as is the case in the rabbit (5). An autosomal recessive gene is the basis for the condition. The gene arose presumably by a spontaneous mutation in a laboratory stock of the house mouse.

The order Sirenia includes 2 living families of aquatic placental mammals, the Manatidae or manatees and the Halicoridae or dugongs. The members of both families are remarkable for their exceedingly dense and massive bones. The medullary cavity is entirely lacking in the long bones and ribs.

Histologic studies on fetal and adult specimens of the Florida manatee made by Fawcett (23) showed that there was a suppression of bone absorption. Fawcett

considered that osteogenesis in Sirenia, in athyreotic states of man and experimental animals, and in marble bone disease of man, has certain features in common: delayed growth in length of bones with relatively normal growth in diameter; incomplete differentiation of periosteal bone into Haversian systems; retardation of endochondral ossification, with disorderly arrangement at the diaphyseocpiphyseal junction; a diminution in number and activity of osteoclasts; unusual persistence of unabsorbed calcified cartilage and primary non-lamellar bone; and a reduction in the amount of functional bone marrow. There also was evidence of an inherent hypofunctioning of the thyroid gland. Because of the similarity of the microscopic structure of the bone in marble bone disease of man, to those in sirenian bone and in skeletons of athyreotic animals, Fawcett suggested a possible endocrine basis for all three conditions.

The descriptions of the skeletal abnormalities in the grey-lethal mouse suggest many points of resemblance to osteopetrosis of the rabbit. Furthermore, both conditions have a lethal effect and both are inherited on the basis of a single recessive gene which in both cases arose as a presumably spontaneous mutation. The analogy between osteopetrosis of the rabbit and the severe infant and juvenile form of marble bone disease in man has already been drawn and it seems not unlikely that the grey-lethal mouse may belong in the same category.

In the case of Sirenia, however, the skeletal peculiarity is a characteristic feature of the species and must be considered a normal, not an abnormal, condition. How close a comparison may be drawn with the benign adult form of human osteopetrosis is not clear but except for certain skeletal changes, there is actually little resemblance to hereditary lethal osteopetrosis of the rabbit or to the severe osteopetrosis disease of infants and young children.

SUMMARY

The manifestations and course of an hereditary disease of the rabbit are reported. The condition is present at birth and is invariably fatal, generally in the 4th and 5th weeks of age. Retardation and eventual cessation of growth with marked reduction in size are conspicuous characteristic symptoms.

The condition, which first occurred in the backcross progeny of a pure bred Dutch male rabbit, is inherited. It is determined by the expression of a simple recessive unit factor, affected individuals being homozygous for the factor. Rabbits heterozygous for the factor are identified only by appropriate breeding tests. The condition is not sex-linked.

The disease has a remarkable resemblance to osteopetrosis or marble bone disease of infants and children with respect to signs and general course and also, as may be stated in anticipation of later discussions (5, 6), to the characteristic abnormal condition of the skeleton.

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EXPLANATION OF PLATES

All illustrations are reproduced from untouched photographs made by Mr. J. A. Carlile.

PLATE 33

FIG. 1. The incisor teeth of a normal rabbit and a litter mate with osteopetrosis 2 days old. Each weighed 65 gm. and was in excellent condition. In the diseased rabbit on the right no upper incisors were visible and only tiny points were palpable through the gum. The slightly separated lower incisors were convergent. The right lower was approximately normal; the left lower was a small very slanting tooth placed slightly posteriorly. $\times 1.84$.

FIG. 2. The incisor teeth of a normal rabbit and an osteopetrosis litter mate 6 days old. Both were in excellent condition; the normal weighed 132 gm. and the diseased animal 112 gm. In the osteopetrosis rabbit on the right the right upper was a small slanting tooth with an opaque slightly bluish white color. At birth, both uppers were erupted; they were small teeth and the shorter left tooth was missing on the 4th day. The lower incisors were also erupted at birth but they were slightly smaller than normal. The difference in size persisted and the teeth became convergent. Their distal half was an opaque yellowish white while the proximal portion was a semiopaque grey. $\times 1.04$.

FIG. 3. The incisor area of a normal rabbit and of an osteopetrosis sib 9 days old. Both were in good condition; the normal weighed 200 gm. and the diseased animal 154 gm. In the osteopetrosis rabbit on the right all 4 incisor teeth were missing. At birth, the left upper was missing and the right was a very small short tooth but the lowers appeared to be normal. These 3 teeth were shed between the 6th and 9th days. $\times 1.04$. X-ray photographs of the jaws and other bones of this animal are shown in the following paper (5, Fig. 5).

FIG. 4. The incisor teeth of an osteopetrosis rabbit aged 13 days. The body weight was 140 gm. and the general condition was fairly good. Both upper incisors were missing from birth. There was also no right lower incisor and the left was small and poorly developed. At birth, both lowers were short and small and the shorter right tooth was shed between the 4th and 6th days. $\times 0.49$. Skeletal x-rays of this rabbit appear in the following paper (5, Figs. 9 and 10).

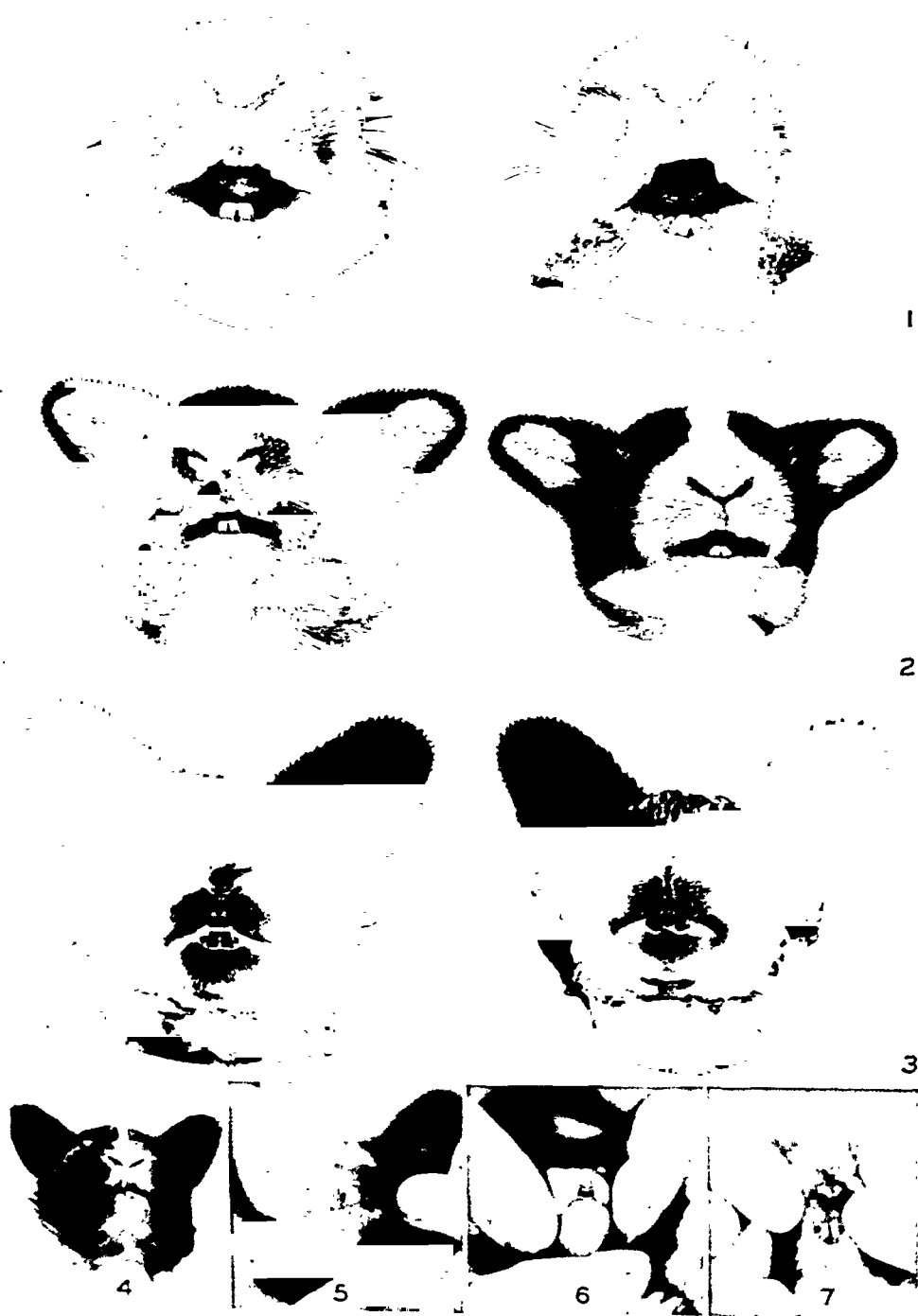
FIG. 5. The incisor teeth of an osteopetrosis rabbit aged 16 days, weighing 294 gm. and in good condition. The point of the right upper could just be felt deep in the gum; the left upper was a tiny thin sliver-like tooth. The lower incisors were very small frail short teeth, quite widely separated and slightly convergent. $\times 0.50$.

At birth, there was a small right upper incisor but the left upper was not seen or felt; the lower incisors had a normal appearance. By the 7th day, the right upper was shed and the lowers were developing very slowly. Toward the end of the 2nd week, the point of the left upper appeared but there was comparatively little growth of the lower teeth. The appearance of this animal on the 16th and 24th days is shown in the photographs in Figs. 11 and 12. It was killed with chloroform on the 27th day and an x-ray photograph of the anterior skeleton is shown in the following paper (5, Fig. 17).

FIG. 6. The incisor teeth of an osteopetrosis male rabbit, aged 21 days, weighing 280 gm., well nourished, and growing. The upper incisors were still of infantile proportions; they were practically perfect in form but their position was twisted, the right being rotated upward and the left downward. The lower incisors were minute peg-like, separated teeth. There had been practically no change in the appearance of all 4 teeth since birth except as regards changes in position relative to one another. The animal died 15 days after this photograph was taken. $\times 0.50$.

FIG. 7. The incisor teeth of a rabbit with osteopetrosis tarda aged 35 days. The slanting right upper was a short stumpy tooth widely separated from its smaller somewhat pointed mate. The separated lowers were abnormally long. The enamel of all 4 teeth was dull, opaque, and very white.

At birth, the incisors appeared normal but at 3 days of age, the uppers were small and separated and at 10 days were tiny fragile teeth. By the 16th day, the left upper was missing and the lowers were thin, long, slightly curved, and separated. At 30 days, both sets were growing and continued to do so, although the general physical condition rapidly deteriorated. Death occurred a few hours after this photograph was taken. $\times 0.63$.



Pearce and Brown. Hereditary osteopetrosis of rabbit. 1.

PLATE 34

FIG. 8. Photograph of a rabbit with osteopetrosis on the left and a normal litter mate on the right aged 7 days. The respective body weights were 148 and 190 gm.; at birth the affected rabbit was slightly the heavier, the weights being 57 and 50 gm. respectively. Other than the small size and the condition of the teeth there was nothing unusual in the general appearance of this animal. The state of nutrition was excellent and the coat was thick, uniform, and sleek. The upper incisor teeth were missing while the right lower was slightly shorter than the left lower. $\times 0.27$.

FIG. 9. The same rabbits shown in Fig. 8 at 15 days of age; the diseased rabbit weighed 224 gm. and the normal litter mate 286 gm. The difference in size had now become well marked but the nutritional state of the osteopetrosis rabbit was still good and growth was continuing. The condition of the coat was also good. The eyelids happened to be closed in the photograph. The upper incisor teeth could still not be seen nor felt but development of the lower incisors was progressing well. $\times 0.27$.

FIG. 10. The same rabbits shown in Figs. 8 and 9 at 27 days of age. The size and weight differences were now pronounced; the osteopetrosis animal weighed 320 gm. and the normal litter mate 530 gm. The diseased animal was still lively and active and the nutritional state was still comparatively good but the coat had become somewhat fluffy and dull and the lacrimal secretion was slightly excessive and somewhat thickened. $\times 0.27$.

The points of the upper incisors were first seen 5 days before this photograph was taken but in the interval, very little growth had occurred. The lower incisors were rather long narrow slightly separated teeth, of an opaque dull yellowish white color. For about 10 days after this photograph was taken, the general condition continued to be fair and the body weight increased to 374 gm. But on the 39th day the weight had decreased to 356 gm. and death occurred 2 days later.



8



9



10

PLATE 35

FIG. 11. Photograph of a rabbit with osteopetrosis on the right and a normal litter mate on the left aged 16 days. The body weights were 294 and 420 gm. respectively; at birth the diseased rabbit weighed 65 gm. and the normal sib 74 gm. The diseased animal was lively and still in a good state of nutrition and growing. The coat was normal. The lacrimal secretion had increased in amount and was more viscous than normal and there were small gummy crusts on the eyelids. The lids were sealed until just prior to the time the photograph was taken. The condition of the incisor teeth on this day is shown in Fig. 5. $\times 0.24$.

FIG. 12. Photograph of the same rabbits shown in Fig. 11 at 24 days of age. The normal litter mate on the left weighed 592 gm. The diseased rabbit on the right weighed 318 gm.: the general physical condition was still fairly good but an indication of the progress of the disease is indicated by body weight values. The peak weight of 336 gm. was reached on the 20th day and was maintained for 2 days; thereafter there was a progressive loss of weight amounting to 18 gm. on the 24th day, when this photograph was taken, and a further 10 gm. during the next 3 days when the animal was killed with chloroform. The photograph shows the somewhat droopy eyelids, dull cornea, and the accumulation of tiny crusts on the lids. The coat was beginning to be rough, irregular, and dull and there was some fecal staining about the anus and external genitalia. $\times 0.24$. The appearance of the incisor teeth was essentially unchanged from that shown in the photograph of Fig. 5 taken at 16 days of age. X-ray films of the anterior skeletons at 27 days appear in the following paper (5, Figs. 17 and 18).

FIG. 13. Photograph of a rabbit with osteopetrosis on the right and a normal litter mate on the left aged 34 days. The normal sib weighed 640 gm. and was growing well. The much smaller osteopetrosis animal was in the terminal stage of cachexia; the body weight was 222 gm. The highest recorded weight of this animal was 271 gm. on the 25th day; thereafter there was a steady loss of weight. During the week prior to the day this photograph was taken, the loss had amounted to 35 gm. while the normal litter mate had gained 198 gm. There were marked weakness and apathy, and an excessive lacrimal gummy secretion, first noted at 2 weeks of age, had become pronounced. An edematous swelling of the external genitalia developed at 3 weeks of age, persisted for several days, and subsided. The coat changes in this animal were comparatively minor. None of the incisor or the molar teeth was erupted and none could be felt through the gum. $\times 0.24$.



HEREDITARY OSTEOPETROSIS OF THE RABBIT

II. X-RAY, HEMATOLOGIC, AND CHEMICAL OBSERVATIONS

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PLATES 36 TO 40

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The signs and course of hereditary osteopetrosis in the rabbit have been described in the preceding paper (1). The disease is present at birth and is characterized by retardation of growth, progressive malnutrition, anemia and cachexia, and a fatal termination usually in the 4th or 5th week of age. Of particular significance are marked bone changes which are a constant feature of the condition.

The investigations on the disease included x-ray examinations of the skeleton, hematologic examinations, and blood and tissue chemical determinations. The results of these studies are reported in the present paper. Postmortem and histologic observations are presented in a subsequent paper (2).

Materials and Methods

The present study is based on 293 rabbits with osteopetrosis and 722 normal litter mates contained in 163 litters together with other normal related and unrelated rabbits of comparable ages.

X-ray photographs of the skeleton of the majority of cases were taken at the time of death, first, for the purpose of confirming the diagnosis and, second, for providing material for study in connection with other observations (1) and with the postmortem and histologic findings (2). A typical series of x-ray photographs of cases at successive stages of the disease is reproduced in this paper. These photographs with a single exception, and all those of the normal litter mates, were taken immediately after the animals had been killed with chloroform.

Hematologic examinations were carried out on a group of osteopetrotic rabbits and normal litter mates; in a few instances normal animals of the same age and stock but from different litters were used. Both single and repeated counts were made. The single counts were carried out on 52 cases and 47 normal rabbits respectively. There were 45 repeated counts on 17 cases and 84 on 47 normal rabbits. The age of the rabbits ranged from 1 to 39 days.

Blood chemical examinations were carried out on 55 cases and 58 normal litter mates at ages of from 1 to 36 days. The blood was obtained by cardiac puncture from chloroform-anesthetized animals. Serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method (3). Serum inorganic phosphorus, and the phosphatase activity of the serum were determined by Bodansky's methods (4). The Folin-Wu method (5) was used for blood sugar determinations and the Sackett method (6) for blood cholesterol.

Liver and muscle glycogen determinations were made on 45 osteopetrotic rabbits and 30 normal litter mates ranging in age from 1 to 36 days. Bollman's modification of Pflüger's method (7) was employed; the tissue was removed immediately after death.

The hematologic and chemical results were analyzed on the basis of the mean values for successive age groups, using intervals of 3 days. This method of analysis was adopted for two principal reasons. First, information on the general trend of these factors in relation to disease progression was desired. Second, the use of mean values minimized individual animal variation. It was found that with the age distribution of the determinations available, mean group values at successive 3 day intervals gave a generally good idea of the results. Some groups were small although the total number of examinations was fairly large. As was to be expected the results were more irregular for the osteopetrosis than for the normal groups and there were instances of considerable variation within a group.

The care and feeding of the rabbits in this colony are elsewhere described (1).

RESULTS

The characteristic condition of the skeleton of rabbits with hereditary osteopetrosis as revealed by x-ray examination will first be described. The x-ray photographs of 13 representative cases at various ages from birth to 34 days together with those of normal litter mates appear in chronological order in Figs. 1 to 20 inclusive. The progress and particular character of the disease are indicated by the legend notes on the general physical condition and the age of the animal.

The next section contains the hematologic observations, and the third, the results of blood and tissue chemical determinations. These data also are presented from a chronological standpoint.

X-Ray Examination of the Skeleton

The typical features of skeletal change as shown in x-ray photographs have been found in all cases from birth throughout life, the average duration of which is 4 or 5 weeks. All the bone shadows of the younger cases show an intense uniform density with practically no detail. In older animals, beginning at about 2 weeks of age, the shadows are less homogeneous and gradually their central portions become more or less translucent but except for the small bones of the feet, the appearance does not become normal. Also to be noted is a size reduction of the skeleton and particularly a shortening of the long bones. It should further be noted that there is some variation in the degree of involvement of different bones and in the time of development and the eventual degree of shadow differentiation.

At birth, as is seen in Fig. 1, the chief feature of the bone shadows is a homogeneous "solid" or opaque appearance without finer structural detail. None of the bones with the partial exception of the vertebrae and sternum shows any suggestion of central cavity differentiation and the shadows are intense and uniform. This abnormality is in striking contrast to the well formed and relatively large marrow spaces of all the bones of normal new-born rabbits (Fig. 2).

It was observed that at birth, general bone development was somewhat less advanced than in the normal rabbit. An indication of this condition is found in the differences in the epiphyseal shadows of the long bones of the x-ray photographs in Figs. 1 and 2.

The bones of new-born osteopetrosis rabbits are well modeled and show no disproportion

of size as is the case in hereditary achondroplasia or chondrodystrophia foetalis of the rabbit (8-10).

In all cases the bones of the skull vault at birth were characteristically fibrous with a variable amount of calcification. In the present case, deficient calcification of the calvarium is well shown (Fig. 1).

During the first days of life, growth and development of the osteopetrosis rabbit occur and the skeleton, in spite of its markedly abnormal condition, participates in these changes although to a variable degree.

An example of the x-ray appearance of the skeleton at 4 days of age is shown in Fig. 3 and one of a normal litter mate appears in Fig. 4. The diseased rabbit was well nourished and in excellent condition. It was smaller than the normal sib at birth, 55 gm. as compared with 70 gm., and the increase in its body weight at 4 days was 64 per cent as compared with a 100 per cent gain for the normal litter mate. From the measurements obtained from the x-ray films, it was found that in the case of the femur and tibia, the lengths of the diseased bones were 67 and 75 per cent respectively of the lengths of the corresponding normal bones and for the humerus and radius, 60 and 77 per cent respectively. These several values are roughly proportional to the difference in the body weight of the two animals, that is, the weight of the diseased rabbit, 90 gm., represents 64 per cent of the weight, 140 gm., of the normal litter mate.

The characteristic dense homogeneous shadow of the bones was present at 4 days (Fig. 3) but a few tiny less intense areas in the central portions may be seen. None of them, however, is comparable to the large well developed marrow spaces of the normal rabbit (Fig. 4). The shape of the bones in the diseased animal is generally well preserved but there is a suggestion of expansion of the ends of the long bones and of the vertebral ends of the ribs.

With increasing age, continued although delayed skeletal growth occurred. In x-ray photographs, differentiation of bone structure begins to appear but the homogeneous dense appearance of the bone shadows is still the outspoken feature.

An example of a typical case and a normal litter mate at 9 days of age are illustrated in Figs. 5 and 6. The diseased animal was growing and apparently in good condition. It will be noted that comparatively little differentiation of structure can be discerned in the femoral and pelvic bones. There is, however, a faint suggestion of marrow cavity formation in the tibia and the bones of the forearm and the central areas elsewhere, particularly in the ribs, sternum, vertebrae, and bones of the feet, show some differentiation. In no bone, however, is the appearance at all comparable with that of the corresponding bone of the normal litter mate.

Typical examples of the bones of 2 cases at 13 days of age are illustrated by the x-ray photographs of Figs. 7, 9, and 10. The nutritional state of both rabbits was good; the animal of Fig. 7 was growing but the body weight of that of Figs. 9 and 10 had not increased for 4 days.

The dense homogeneous bone shadows in Fig. 7 are perfectly characteristic and are very similar to those of the 9 day old case (Fig. 5) just described. Both the anterior and posterior ends of some of the ribs are somewhat expanded; the ends of the femur, tibia, and humerus are also moderately expanded and the central portions of the shafts are slightly constricted.

In the second 13 day case, the x-ray photograph (Fig. 9) shows that the marrow cavity differentiation of the leg bones was slightly more advanced than in the other case (Fig. 7). There is a considerable expansion together with a pronounced shadow intensification of the anterior ends of the ribs. The ends of the long bones are slightly expanded and the central portions of the shafts, and particularly that of the femur, are somewhat constricted. There is an additional feature in this case which is occasionally found, namely, an indentation or depression of some of the ribs; in this animal the anterior portions of the 8th and 9th right ribs were affected. The condition is faintly suggested in Fig. 9 but it is well shown in the x-ray of the dissected bones in Fig. 10. Also well shown in the latter photograph are the dense bulbous ends of the ribs and the homogeneous dense shadows of the sternal bones. These abnormal features are in sharp contrast to the x-ray findings in a normal rabbit, an example of which is included in the same illustration. The appearance of the bodies of the diseased ribs (Fig. 10) suggests that a moderate differentiation of marrow cavity had taken place.

Three cases at 15 days of age will now be described. The first presented some unusual features which are disclosed by the x-ray photograph in Fig. 8.

Growth of this rabbit had been greatly retarded, the birth weight only having doubled in a fortnight, but in spite of evident malnutrition, the animal was comparatively lively up to the day it was found drowned in the water trough. In the x-ray photograph most of the bones have a characteristic dense shadow with small irregular areas of differentiation. Both femora show an extreme shortening together with a marked wasp-like constriction of the central portion of the shafts. The ends of these bones are expanded. In the tibia and to a less extent in the bones of the upper extremity, there is a suggestion of similar changes but in none of them does the degree of shortening approach that of the femora. Hydrocephalus which was suspected during life was found at postmortem examination. There was a marked calcification deficiency of the bones of the calvarium and as is seen in the x-ray (Fig. 8) the posterior fontanelle had not closed. The bones were brittle as was discovered when the right humerus was accidentally fractured at autopsy.

The other 15 day cases are represented by portions of the dissected skeletons of 2 litter mates shown in Figs. 11 and 12. Also included are the photographs of a front extremity and the 9th ribs and vertebra of a normal litter mate (Fig. 12). Both diseased rabbits were in good physical condition and were growing. It will be noted that in both cases, some differentiation of long bone structure had occurred and that it was more advanced in the rabbit of Fig. 12 than in the one of Fig. 11. A comparison with the normal bones, however, shows that this process was by no means complete. The appearance of the ribs in the photograph of Fig. 11 should be noted. The stage of marrow cavity differentiation is not very advanced and the intensity of the cortical shadow is not marked. There was practically no expansion of the rib ends of this case.

The x-ray appearance of the bones of older cases will now be described.

Fig. 13 shows the bones of a 21 day old rabbit and Fig. 15 of another with osteopetrosis aged 24 days. The condition of normal bones at the latter age is illustrated by the x-ray of a normal litter mate (Fig. 16). The physical condition of the 21 day old rabbit was fairly good but there had been very little gain in body weight for 4 days. The 24 day old rabbit was in comparatively poor condition. There was a rapidly progressive cachexia, a loss of body weight, and diarrhea which had developed quite suddenly 3 days previously.

The bone shadows of both these diseased rabbits are typically dense although there are small ill defined central areas of lessened intensity particularly in the foreleg bones of the first case (Fig. 13) and in the femur and tibia of the second case (Fig. 15). Some degree of marrow

cavity differentiation is also distinguishable in other bones of both cases although in comparison with normal bones (Fig. 16), the change seems insignificant. In both cases, the ends of the leg bones show some expansion and the shafts, some constriction. Calcification of the calvarium, especially of the 24 day old case (Fig. 15), was deficient and beading of the anterior ends of the ribs was prominent in the 21 day old case (Fig. 13).

A conspicuous feature of the 24 day old case (Fig. 15) was the small size of the bones; the contrast with a normal litter mate (Fig. 16) is very marked. This rabbit was an example of the so called tarda case in which the disease was not suspected at birth because the incisor teeth appeared to be normal. At 7 days of age, however, the upper incisors had shown very little growth, the right lower incisor was missing and the left lower had become a frail thin tooth. Hydrocephalus was suspected on the 10th day and was thought to be definite on the 13th day. For 3 weeks, the general condition of the rabbit was good and there was a constant although comparatively slow gain of body weight. On the 21st day, there was a loss of body weight and the rabbit was weak and listless and on the following day, diarrhea was present. These signs increased very rapidly and on the 24th day the precarious condition of the animal was evident and it was killed. At autopsy, the smallness of the skeleton and particularly the reduced length of the long bones was striking. In addition, the characteristic appearance of the split leg bones and the increased hardness and brittleness of the bones generally were typical (2). Hydrocephalus was not found but the calvarium showed deficient calcification as is indicated in the x-ray photograph (Fig. 15).

The degree of size reduction of the bones of this 24 day old case will be appreciated by a comparison of the x-ray photograph (Fig. 15) with that of a normal litter mate of the same age (Fig. 16). In this connection the character of the growth of both animals as indicated by body weight records is of interest. At birth, both the diseased and the normal rabbit weighed 40 gm. At 24 days of age, the respective weights were 156 and 324 gm. In terms of the birth weights, the gains of 116 and 284 gm. represent increases of 290 and 710 per cent respectively. The final weight of the osteopetrosis rabbit was 48 per cent of the final weight of the normal sib. Measurements of the femur and tibia obtained from the x-ray photographs show that the length of the osteopetrosis bones was approximately two-thirds that of the corresponding normal bones.

In still older cases, the x-ray photographs continue to show typical dense bone shadows although greater degrees of differentiation are usually present. Three cases aged 31, 27, and 34 days respectively have been selected to illustrate the x-ray appearance of the skeleton in the late and terminal stages. In the average survival period of 4 to 5 weeks, increasingly severe signs of malnutrition, loss of weight, emaciation, weakness, and intestinal disturbances indicate the progressive course of the disease.

The 31 day old case illustrated in Fig. 14 was an example of a relatively infrequent form of the disease in that the body weight was still increasing and the general physical condition was still good in the 5th week of age. Growth had been retarded, however, and the size of the rabbit was only about half that of 2 normal litter mates, the respective body weights being 340, 640, and 690 gm. It will be noted in Fig. 14 that a considerable differentiation of bone structure had taken place with the production of quite well defined central cavities. The change is most marked in the radius and ulna and in the bones of the sternum and feet, but it is present in greater or less degree in the other bones. It is least marked in the femur and ilium.

The x-ray photograph of the anterior skeleton of a 27 day old case is shown in Fig. 17 and that of a normal litter mate in Fig. 18. Photographs of these 2 rabbits at 16 and 24 days of

age appear in the preceding paper (1, Figs. 5, 11, and 12). The general condition of the osteopetrosis rabbit was still fairly good on the 27th day but 4 days previously a loss of body weight had been found and on the day the animal was killed, the total loss amounted to 28 gm. from the peak weight of 336 gm. attained on the 20th and 22nd days. The x-ray appearance of the bones (Fig. 17) is typical and is comparable to that of other older rabbits. The bones show the usual size reduction. A considerable degree of marrow cavity differentiation has taken place generally, particularly in the radius and ulna. The cortical shadows of the long bones are quite prominent and are somewhat wider than those of the normal litter mate (Fig. 18). The expansion of the vertebral ends of the ribs, particularly of the 8th, 9th, and 10th ribs is well marked and there is not much contrast between the cortex and the central portions of the ribs generally.

The x-ray photograph of the posterior skeleton, the base of the skull, and the sternum of a 34 day old case is shown in Fig. 19; a similar x-ray of a normal litter mate of the same age appears in Fig. 20. This case was an example of unexpected survival for some 10 days after the development of malnutrition and weakness. The signs increased quite rapidly and on the 30th day, diarrhea developed. On the 34th day, the rabbit was prostrated and practically moribund. The weight records are of interest. At birth the weight was 52 gm.; on the 14th day, 200 gm.; on the 21st day, 250 gm.; on the 34th day, 230 gm. The normal litter mate weighed only 30 gm. at birth; on the 14th day, 220 gm.; on the 21st day, 360 gm.; and on the 34th day, 646 gm.

The most conspicuous feature of the x-ray of this 34 day old osteopetrosis rabbit is the marked stunting of the bones (Fig. 19). The change is pronounced in the femur, tibia, and pelvis and only slightly less so in the case of the feet, the sternum, base of the skull, maxilla, and vertebrae. Both ends of the femur and tibia are expanded and the central portions of the shafts and especially of the femur, are constricted. The shadows of the vertebrae and of the pelvic and leg bones indicate that some differentiation of central marrow spaces has taken place. This process is more advanced in the sternum and the metatarsal and phalangeal bones. The base of the skull and the maxilla show deficiency of calcification.

Hematologic Observations

The principal hematologic characteristics of osteopetrosis of the rabbit are macrocytic anemia, thrombocytopenia, and moderate myeloid leucocytosis. These and other findings will now be described in terms of the mean group values at successive 3 day intervals from 1-3 to 37-39 days of age (Charts 1, 2). The details of repeated blood examinations of 2 cases are also presented as examples of the results in individual rabbits (Table I).

The mean *red blood cell* counts for the first week or 10 days of life tended to be slightly higher than those of normal litter mates (Chart 1). Thereafter, the mean values gradually became lower while the corresponding normal values rose, at first rather slowly, but after the 3rd week, quite rapidly. By the end of the 5th week, the mean red cell count of the osteopetrosis rabbits was approximately 3 million per c.mm. as compared with 5 million per c.mm. for the normal litter mates.

The mean *hemoglobin* values of the rabbits with osteopetrosis were comparable, although at slightly higher levels, to those of the normal rabbits for the first 3 weeks of life (Chart 1). The curves representing both groups of rabbits describe parallel falls from the initial high value of approximately 11.85 gm. to the level of 8.13 gm. in the 19-21 day interval. Further declining values were a feature of the older osteopetrosis groups, in contrast to higher levels found in the older normal animals. In the 37-39 day interval, the values were 5.42 and 9.15 gm. for the osteopetrosis and the normal rabbits respectively.

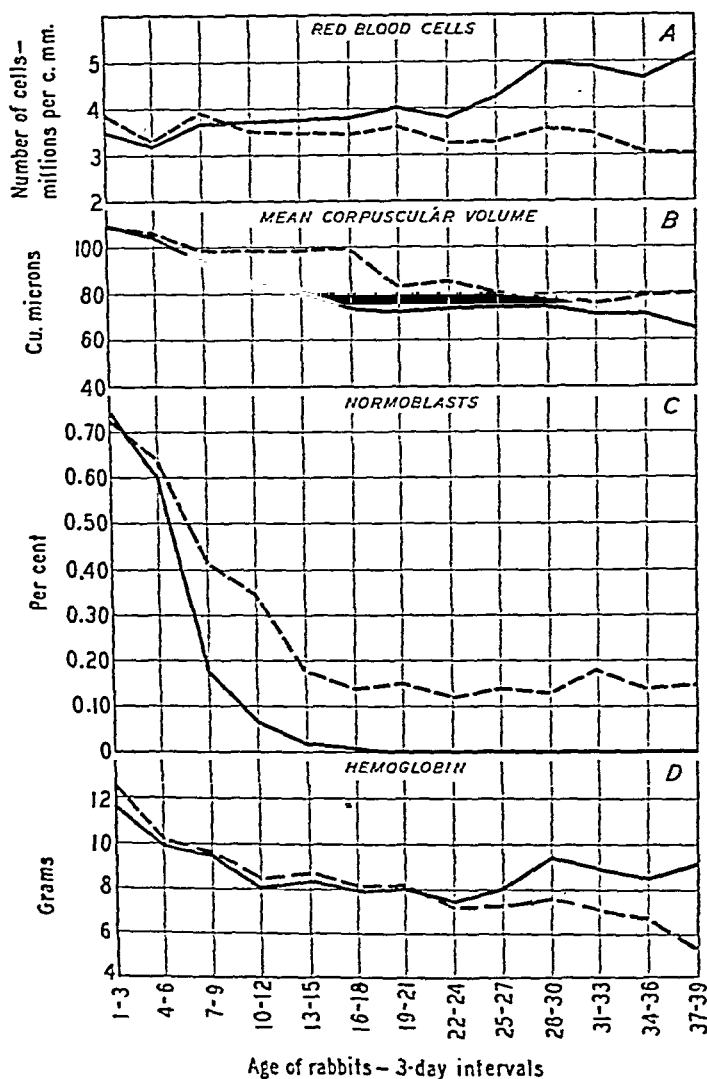


CHART 1. Mean values of erythrocytes and the mean corpuscular volume, normoblasts, and the hemoglobin content for groups of osteopetrosis and normal litter mate rabbits. In this and all other charts the values for the osteopetrosis groups are represented by broken lines and those for the normal groups by solid lines. Similarly all time intervals are 3 days.

The results for mean *hematecrit* determinations resembled those for mean hemoglobin values. The mean values of both groups for the 1-3 day interval were at approximately the 40 per cent level; increasingly lower readings followed for 3 weeks, but those of the osteopetrosis groups were at slightly higher levels than those of the normal groups. In the 22-24 day interval, the mean value for the osteopetrosis cases was 28 per cent as compared with 26 per cent for the normal rabbits. During the next 3 weeks, however, the values for the normal

animals rose while those for the diseased rabbits continued to fall, the last mean values in the 37-39 day interval being 24 and 36 per cent for the groups of diseased and normal animals respectively.

The values for *mean corpuscular volume* of the osteopetrosis cases exceeded those of normal litter mates after the 1-3 day interval (Chart 1). The curve representing mean normal values shows a gradual decline from the level of 110 cubic microns for the 1-3 day interval to 65 cubic microns for the 37-39 day interval. Declining values for the osteopetrosis groups also occurred but in a more irregular manner. Stabilization at the level of 100 cubic microns was observed in the 7-18 day period when normal values decreased from 96 to 74 cubic microns. For the diseased groups examined in the 3rd, 4th, and 5th weeks of age, the level ranged from 76 to 86 cubic microns as compared with 65 to 74 cubic microns for the normal groups.

The *mean corpuscular hemoglobin content* for the diseased and normal rabbit groups are represented by the curves in Chart 2. The form of both curves is similar and for the first 10 days they are practically identical. Both describe a sharp fall from an initial mean value during the 1st week of approximately 32 micromicrograms to 21 micromicrograms for the normal animals and 22 micromicrograms for the osteopetrosis rabbits in the 10-12 day interval. Subsequent results for both groups showed a slight trend toward lower levels. The values for the osteopetrosis groups, however, continued to be slightly higher than those for the normal animals, the difference being 1 to 2 micromicrograms.

The *mean corpuscular hemoglobin concentration* for the 1st week of life was slightly higher for the osteopetrosis groups. In the 4-6 day interval, the mean values were 28.4 gm. for the osteopetrosis and 26.2 gm. for the normal group. The results in the 2nd and 3rd weeks of age showed lower mean values for the osteopetrosis group, the range being 25.4 to 28.0 gm. as compared with 26.2 to 29.3 gm. for the normal litter mates. For the groups in the 4th and 5th weeks of age, the mean values for the osteopetrosis cases were higher than for the normal rabbits, the level ranging from 27.1 to 27.8 gm. for the diseased and 23.9 to 25.6 gm. for the normal groups. In the 37-39 day interval, the low value of 22.6 gm. was found for the single osteopetrosis case examined while the mean value of 25.4 gm. for the 4 normal litter mates showed no significant change from the level of the preceding 2 weeks.

The *mean reticulocyte counts* of the diseased rabbits exceeded those of the normal litter mates. In the 1st week, the mean count of both groups varied from 25 to 32 per cent. During the next 3 weeks, the counts fell quite abruptly to the level of 5 to 6 per cent for the normal rabbits and 8 to 10 per cent for the diseased rabbits. In the 4th and 5th weeks of age, the counts for the normal groups were practically unchanged but higher values of from 11 to 14 per cent were observed in the osteopetrosis groups.

Normoblasts were constantly found in the circulating blood of the rabbits with osteopetrosis (Chart 1). In the normal rabbits the curve of the mean relative numbers of normoblasts describes a steep fall from 0.75 per cent to 0.18 per cent in the 7-9 day interval; still smaller numbers were subsequently found up to 3 weeks of age, after which none was observed. The curve representing the osteopetrosis rabbits resembles that for the normal rabbits with two important differences. First, normoblasts continued to be present in all age groups. In those examined at from 2 to 6 weeks of age the mean relative number averaged 0.15 per cent. Second, the rate of decrease was less rapid and abrupt than that observed in the normal groups. An occasional megaloblast was also seen in osteopetrosis blood films whereas no cell of this class was ever observed in normal blood.

Although the erythrocytes of the osteopetrosis cases generally appeared larger than those of the normal rabbits, there were cases in which extremely small red cells were seen scattered about among the larger ones. Poikilocytosis of degrees greater than is found in normal rabbit blood was also observed. The incidence of both anisocytosis and poikilocytosis was variable but they tended to occur more frequently and in more marked degree in older cases. Con-

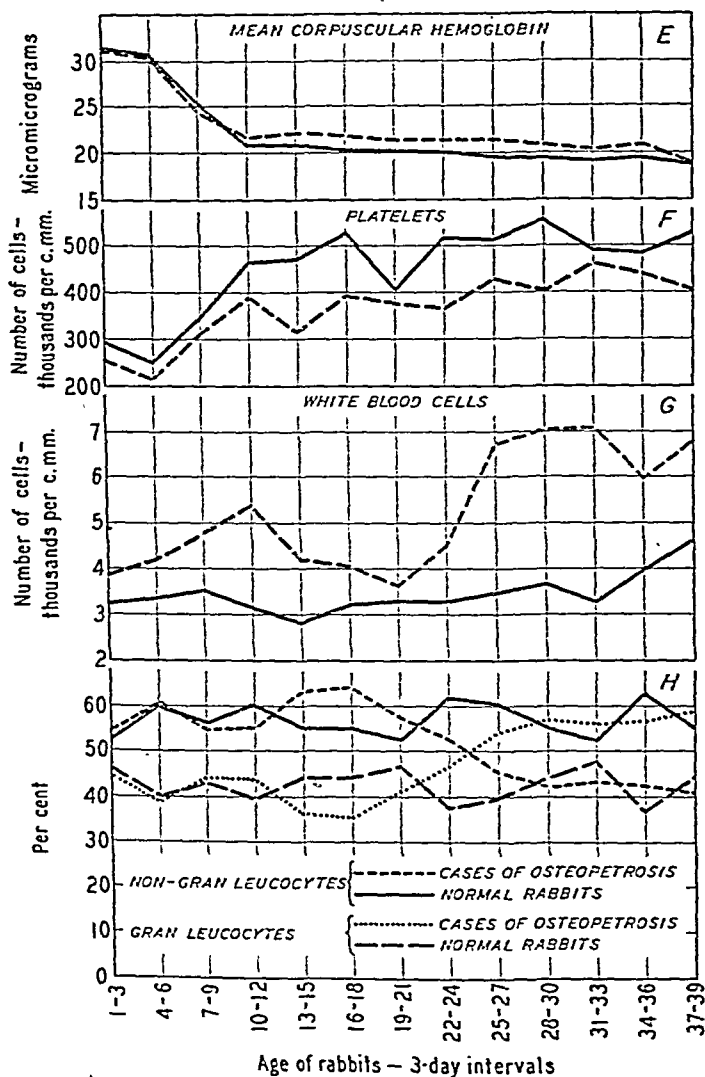


CHART 2. Mean values of the mean corpuscular hemoglobin content of the erythrocytes, the platelets, total leucocytes, and the relative numbers of granular and non-granular leucocytes for groups of osteopetrosis and normal litter mate rabbits.

Considering only very advanced grades, the results of a single examination of 54 cases aged 5 to 35 days showed anisocytosis in 26 cases, or 48.2 per cent, and poikilocytosis in 15 cases, or 27.8 per cent.

The appearance of the erythrocytes of the osteopetrosis rabbits, and particularly the older cases, included other abnormal features. In many cases there was a conspicuous pallor of the central portion of the cells. There was also a tendency toward a persistence of polychromato-

philia and of basophilic stippling well beyond the age of 2 weeks at about which time these features are not commonly seen in normal red cells. They were observed in some cases which lived 4 and 5 weeks.

With the progression of the disease, the erythrocytes tended to show pseudopodia-like processes and crenation, changes not observed in the red cells of normal rabbits. In occasional cases 3 weeks of age and older, the finding of burst or disintegrating red cells suggested increased fragility. A fragility test on 2 diseased and 3 normal litter mates 24 days old gave the following results: for both diseased rabbits beginning hemolysis occurred with 0.46 per cent NaCl and complete hemolysis with 0.36 per cent; for the 3 normal rabbits, beginning hemolysis with 0.40 per cent NaCl and complete hemolysis with 0.30 per cent in the case of 2 and with 0.28 per cent in the case of 1 normal rabbit respectively.

The mean *platelet* counts are illustrated by the curves in Chart 2. The general form of the curves for both classes of rabbits is similar but the general level of the osteopetrosis curve is consistently lower than the normal one. In the 1-3 day interval, the mean platelet count of the osteopetrosis group was 250,000 per c.mm. as compared with 300,000 per c.mm. for the corresponding normal group. With increasing age the mean normal values rose irregularly to 530,000 per c.mm. in the 37-39 day interval. The mean counts of the osteopetrosis cases also increased irregularly to a level of 410,000 per c.mm.

The mean *leucocyte* counts of the diseased rabbits were consistently larger than those of the normal animals (Chart 2). There was a gradual increase in the normal counts from 3250 cells per c.mm. in the 1-3 day interval to 4680 cells per c.mm. in the 37-39 day interval, a gain of 44 per cent. The corresponding mean values for the osteopetrosis cases were 3850 and 6750 cells per c.mm., an increase of 75 per cent. The sharply rising values were abruptly interrupted at the end of the 2nd week, and during the 3rd week much lower levels were observed. This was followed by a marked rise which was sustained with one minor downward fluctuation to the last examination. As is shown in the curves, the changes in direction of successive leucocytic levels for both classes of rabbits were similar but those of the osteopetrosis groups occurred slightly later and were much more pronounced than those of the normal groups.

The mean relative proportions of *granular* and *non-granular leucocytes* are shown in 4 curves (Chart 2). The curves for both classes of cells in the successive normal groups are somewhat irregular but there are no pronounced or sustained fluctuations. The relative numbers of non-granular leucocytes were consistently higher than those of the granular cells. In the osteopetrosis rabbits, the relative numbers of non-granular and granular leucocytes were approximately the same as normal values in the groups examined in the first 2 weeks of life but in the older groups, increasingly higher values for granular leucocytes and correspondingly lower values for non-granular cells were found. The rise in granular cells pursued a practically uninterrupted course with no reversal in trend. For the last 4 observation intervals, 28-39 days, the average level of granular leucocytes for the osteopetrosis groups was 58 per cent as compared with 44 per cent for the normal groups. An occasional older case failed to develop a granular leucocytosis.

The results of differential white cell counts of osteopetrosis rabbits showed no significant deviations from normal values in the proportions of eosinophiles, basophiles, and monocytes. Likewise, the relative numbers of small and large lymphocytes were essentially normal, the proportion of approximately 10 per cent of large lymphocytes being maintained.

Several interesting abnormal features in the leucocytes of osteopetrosis cases were observed in stained blood films. First, the majority of the neutrophils were young cells, the predominant type of nucleus having 1 or 2 lobes. Second, myelocytes were seen in 75 per cent of the cases and an occasional myeloblast was found. Third, basophilic degenerative changes of the neutrophils were seen in about 20 per cent of the cases. In most of them, approximately one-quarter or one-third of the neutrophils were affected; in a few cases almost all the cells

showed the change and in some cases only an occasional degenerated cell was seen. Both the nucleus and the cytoplasm, or the nucleus or the cytoplasm, were affected and there was about an equal representation of these three types. The basophilic granules in the nucleus were essentially the same color as those in the cytoplasm but they were definitely larger; on the average, there were 2 of these bodies but as many as 3 have been seen. In a few of the cases similar granules were found in the nucleus, but not in the cytoplasm of the lymphocytes. There was no apparent relation between the occurrence of this basophilic degeneration and the age or the condition of the animal. It has been observed in cases 4 days old and if it was found in the first examination, it was always present in subsequent ones. No instance of basophilic toxic granulation was observed in the eosinophiles or the monocytes of the diseased rabbits nor in any of the blood cells of the normal rabbits.

Two other types of leucocytic degeneration were noted. In a few cases, approximately 2 per cent, toxic vacuoles were seen in the cytoplasm of some of the neutrophiles and lymphocytes. In a larger number of cases, upwards of 15 per cent, the presence of nuclei from ruptured leucocytes was conspicuous. In some films such nuclei comprised 4 or 5 per cent of the white cells whereas in normal blood, only 1 or 2 in several hundred white cells were found.

In a small proportion of cases, approximately 5 per cent, there was a noticeable variation in the size of all the leucocytes, both granular and non-granular. Enlargement was observed somewhat more frequently than reduction and in some specimens, the increase in total area amounted to fully twice that of a normal cell. The degree of reduction in size was less marked. In both types of size variation, both the nuclei and the cytoplasm were affected.

The results of repeated hematologic examinations on 2 typical cases with their 2 normal litter mates will now be described. It will be noted that the findings resemble in all essential respects the several mean group values just discussed.

The first pair of rabbits was examined on the 13th, 20th, 27th, and 39th days of age (Table I). On the 27th day the diseased animal continued to show a gain in body weight and was in fairly good condition but on the 39th day cachexia was well advanced and there was a marked loss of weight; death occurred on the 42nd day.

The erythrocyte counts of this case were much lower on the 20th, 27th, and 39th days than on the 13th; the final count of the normal animal was higher than the 3 previous ones. Normoblasts were present in all the blood films of the diseased rabbit and in the first film of the normal sib. There was a marked continuous drop in the hemoglobin and hematocrit estimations whereas these values for the normal rabbit were maintained or were increased. All four values for mean corpuscular volume of the osteopetrosis rabbit were larger than those of the normal litter mate and a similar difference was found for the mean corpuscular hemoglobin on the 13th, 20th, and 39th days. The results for mean corpuscular hemoglobin concentration were variable being lower for the osteopetrosis rabbit on the 13th and 27th days and higher on the 20th and 39th days. For both rabbits, the final platelet count was higher than the initial level at 13 days, but that of the osteopetrosis rabbit showed a much smaller relative increase.

The total leucocyte count of the normal rabbit showed a steady rise. The white count of the osteopetrosis rabbit was considerably higher than that of the normal rabbit at 13 days; at 20 and 27 days it had dropped well below its initial figure but at 39 days it had increased to a value much higher than either its initial value or the final count of the normal litter mate.

In the first differential white cell counts, both animals had a higher proportion of granular than of non-granular leucocytes. At 20 days, this ratio continued for the diseased rabbit but for the normal the non-granular cells predominated. At 27 days, non-granular cells

predominated in both animals. At 39 days, the granular leucocytes again exceeded the non-granular in the diseased rabbit while in the normal animal both classes of cells were about equally represented. Leucocytic degeneration was observed in all the blood films of the osteopetrosis rabbit but in none of those of the normal litter mate.

TABLE I

Results of Repeated Hematologic Examinations on Two Cases of Osteopetrosis

Group	Rabbit	Age	Wt.	RBC	Hb	Ht	MCV	MCH	MCHC	Platelets	WBC	N	L	B	E	M	Norm.	Leu. Deg.
		days	gm.	per c.mm.	gm.	per cent	c.μ	γ γ	per cent	per c.mm.	per c.mm.	per cent	per cent	per cent	per cent	per cent		
I	Ost.	13	138	4,340	9.83	48	110	22.6	20.4	380	5,950	48	34	14	1	3	++	+
	Nor.	13	232	4,180	9.32	37	88	22.3	25.2	390	3,100	55	39	4		2	+	
	Ost.	20	217	3,100	7.45	25	80	24.0	29.8	520	3,400	55	43		1	1	++	+
	Nor.	20	370	3,860	7.79	29	74	20.2	26.8	370	3,750	37	63					
	Ost.	27	320	2,900	5.93	22	86	20.5	26.9	370	2,000	38	60	2			+	+
	Nor.	27	522	3,400	7.62	23	67	22.4	33.1	620	4,150	20	76	2		2		
	Ost.	39	265	3,000	5.42	24	80	18.1	22.6	410	6,750	59	40			1	+	+
	Nor.	39	580	5,750	8.81	41	53	15.3	21.4	550	4,900	50	43	2		5		
	Ost.	9	156	3,760	8.64	36	96	22.9	23.9	385	3,950	43	51	6			++	+
	Nor.	9	172	4,370	9.99	38	87	22.8	26.3	490	2,000	36	57	6	1		+	
II	Ost.	19	209	4,240	8.81	34	80	20.7	25.9	470	6,250	55	44	1			+	+
	Nor.	19	272	4,380	8.86	32	73	20.1	27.5	515	2,850	15	79	5		1		
	Ost.	27	176	3,600	8.13	27	75	22.6	30.1	420	11,850	77	23				+	+
	Nor.	27	400	4,850	9.49	34	70	19.6	27.9	480	3,750	24	70	2	1	3		

Ost., osteopetrosis rabbit; Nor., normal litter male; RBC, red blood cells (000 omitted); Hb, hemoglobin (Newcomer); Ht, hematocrit (Van Allen); MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; platelets (000 omitted) (Rees-Ecker); WBC, white blood cells; N, neutrophils (pseudo-eosinophiles); L, lymphocytes; B, basophiles; E, eosinophiles; M, monocytes; Norm., normoblasts; Leu. Deg., leucocytic degeneration.

The blood counts on the 2nd pair of rabbits (Table I) were made on the 9th, 19th, and 27th days of age. On the 19th day, the osteopetrosis rabbit was in good condition and gaining weight but on the 27th day, malnutrition and weakness were well marked and a loss of body weight had occurred. The rabbit was found dead on the 33rd day. The results of the blood examinations were generally similar to those of the other pair and need not be described in detail. The erythrocyte and hemoglobin levels of the osteopetrosis rabbit showed only minor falls and the decrease in hematocrit values was not as great as in the other case. The eventual leucocyte count, however, was considerably larger and the proportion of neutrophile cells much higher. Again, all the values for mean corpuscular volume were larger than the corresponding

normal values, and those for mean corpuscular hemoglobin were slightly or moderately larger. The mean corpuscular hemoglobin concentration of the osteopetrosis rabbit was lower than that of the normal rabbit on the 9th and 19th days but higher on the 27th day. The presence of normoblasts and the incidence of leucocytic degeneration were similar to the findings in the other osteopetrosis rabbit.

Blood and Tissue Chemical Determinations

A limited chemical study included the following determinations: calcium, inorganic phosphorus, and phosphatase of blood serum; sugar and cholesterol

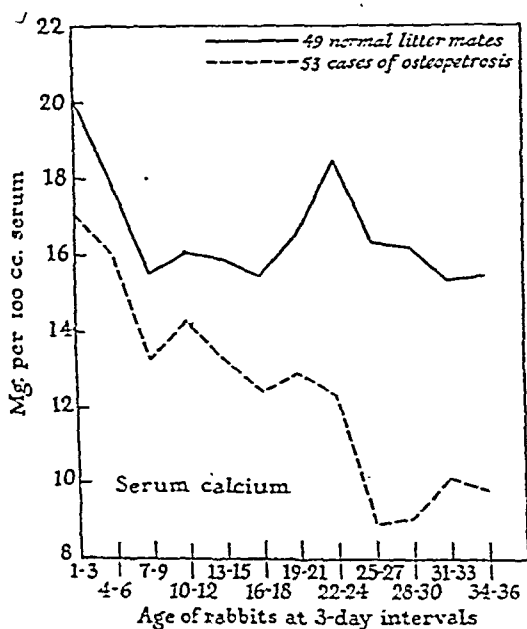


CHART 3. Mean values for serum calcium of 53 osteopetrosis and 49 normal litter mate rabbits.

of whole blood; and liver and muscle glycogen. The results were analyzed on the same basis as the hematologic observations, that is, mean group values for 3 day intervals from birth to 36 days of age.

Serum Calcium.—The calcium content of the serum of rabbits with osteopetrosis was consistently lower than that of normal litter mates (Chart 3). For the normal rabbits the mean value in the 1-3 day age group was 20 mg. per 100 cc.; by the end of the 1st week, and continuing thereafter, a level of 15.5 to 16.5 mg. was maintained, except for an unexplained rise to 18.5 mg. in the 22-24 day interval. For the osteopetrosis rabbits, the highest mean calcium value observed, 17.2 mg. per 100 cc., likewise occurred in the first 3 days of life. Subsequent values showed a continuous although irregular decline toward much lower levels with

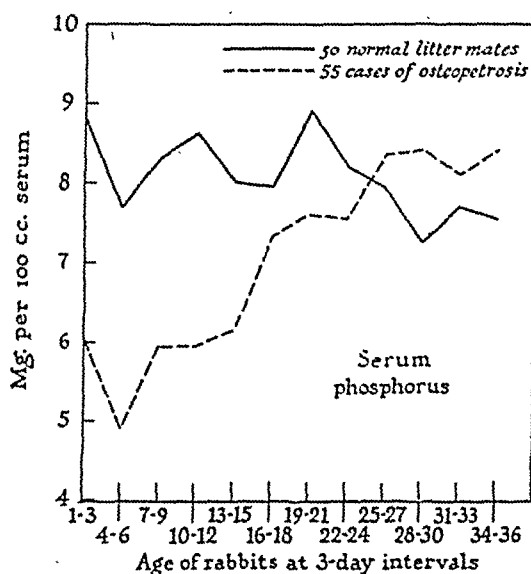


CHART 4. Mean values for serum phosphorus of 55 osteopetrosis and 50 normal litter mate rabbits.

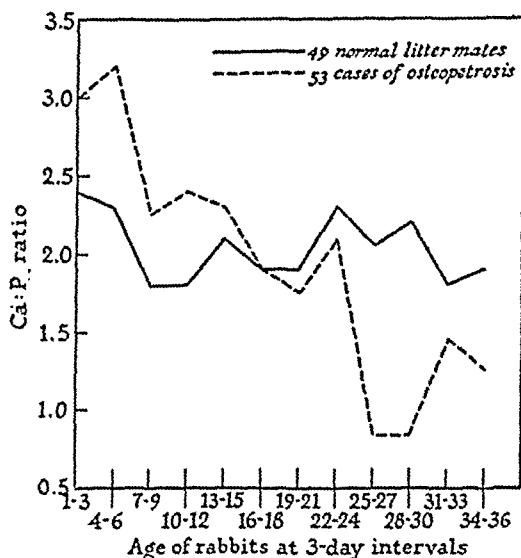


CHART 5. Mean values for the calcium-phosphorus ratios calculated from the respective determinations represented in Charts 3 and 4.

perhaps some stabilization in the 5th week; the last mean value in the 34-36 day interval was 9.8 mg. per 100 cc.

Serum Phosphorus.—The mean serum phosphorus values of the normal rabbits ranged between 8.7 and 7.3 mg. per 100 cc.; the general level was higher in the first 3 weeks than in

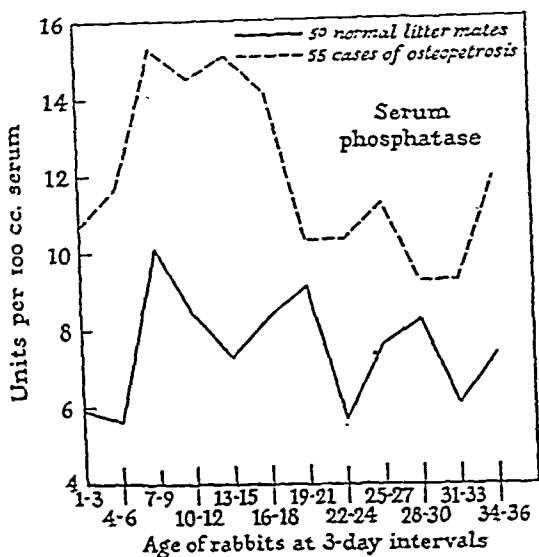


CHART 6. Mean values for serum phosphatase of 55 osteopetrosis and 50 normal litter mate rabbits.

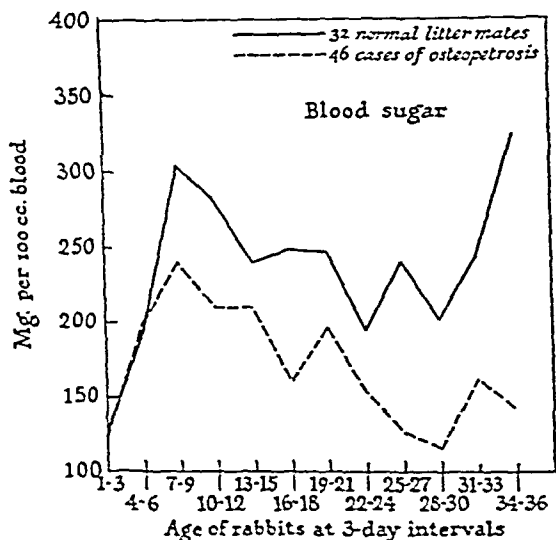


CHART 7. Mean values for blood sugar content of 46 osteopetrosis and 32 normal litter mate rabbits.

the 4th and 5th weeks of age (Chart 4). In contrast to these results the mean values of the younger osteopetrosis cases were lower than those of the older groups. For the first 3 weeks the mean values of the osteopetrosis cases were much lower than those of the corresponding normal groups. From a level of 5.8 mg. per 100 cc. for the 10-12 day group, subsequent values

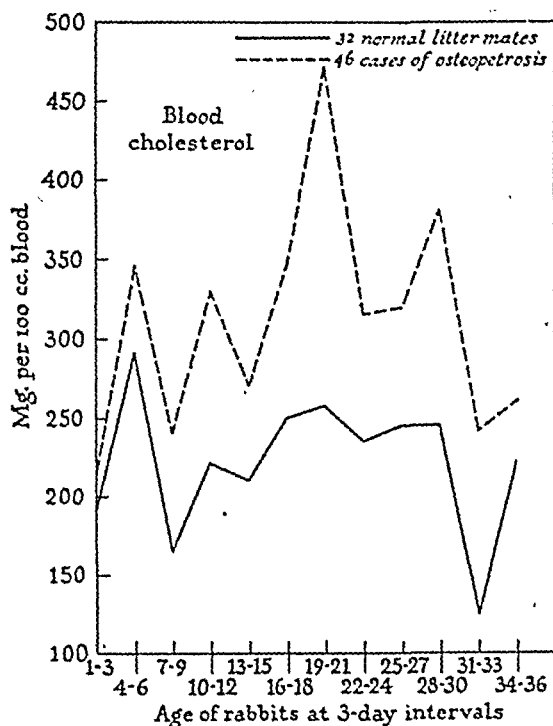


CHART 8. Mean values for the cholesterol content of whole blood of 46 osteopetrosis and 32 normal litter mate rabbits.

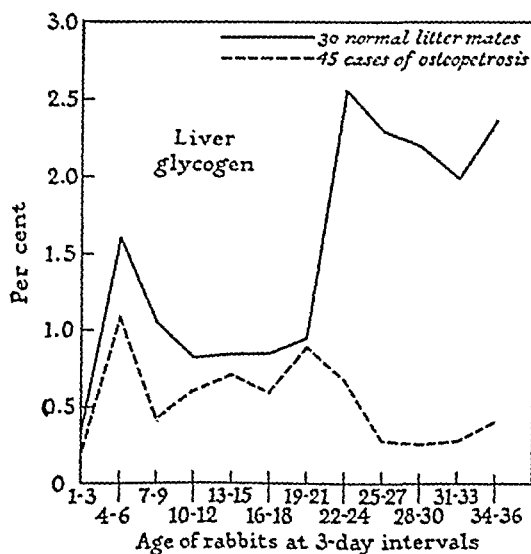


CHART 9. Mean values for liver glycogen content of 45 osteopetrosis and 30 normal litter mate rabbits.

were consistently higher and in the 5th week, they exceeded those of the normal groups. The last mean phosphorus content for the diseased animals was 8.4 mg. as compared with 7.6 mg. per 100 cc. for the normal litter mates.

Calcium: Phosphorus Ratio.—The ratios of the normal groups show some variability but their general level is around the value of 2.0 (Chart 5). The curve representing the osteopetrosis groups describes a progressive decline from the high points of 3.0 and 3.2 for the youngest groups to values ranging from 0.8 to 1.9 for the groups in the 4th and 5th weeks of life. The level of the curve is higher than the curve of the normal rabbits up to the 16–18 day group, but for the older groups it is consistently lower.

Phosphatase.—The mean values for alkaline phosphatase of the normal rabbits ranged from 5.6 to 10.2 units per 100 cc. of serum (Chart 6). The results were irregular but somewhat lower values were found in the older than in the younger groups and in the 5th week, levels of 6.0 to 7.3 units obtained.

Much higher mean values for serum phosphatase were regularly found in the groups of osteopetrosis rabbits and particularly in the 2nd and 3rd weeks, when the level varied from 14.5 to 15.3 units per 100 cc. of serum. As was the case with the normal animals, the lowest values occurred in the older groups and for the 4th and 5th weeks, the mean values varied from 9.2 to 11.5 units.

Sugar.—The mean group values of the sugar content of whole blood in osteopetrosis rabbits were lower than those of the corresponding normal animals except for the 2 youngest groups in which no difference was found (Chart 7). From the 2nd to the 5th week of age the level for the normal animals ranged from 300 mg. to 200 mg. per 100 cc. of blood; in the 34–36 day interval, the mean value was 325 mg. per 100 cc. The corresponding mean values for the diseased rabbits followed a progressively declining trend to the level of 142 mg. per 100 cc. in the 34–36 day intervals.

Cholesterol.—The mean group values for blood cholesterol determinations of the normal rabbits were somewhat irregular but their general level was 225 mg. per 100 cc. of blood (Chart 8). The results for the groups of osteopetrosis cases were much more irregular but all mean values exceeded those of the normal groups. The general level approximated 325 mg. per 100 cc. of blood.

Liver Glycogen.—In the normal rabbits, higher mean values for liver glycogen were found in the older than in the younger groups (Chart 9). The values for the first 3 weeks ranged from 0.35 to 1.6 per cent and in the 4th and 5th weeks rose to a level of from 2.0 to 2.6 per cent. In the osteopetrosis groups, all mean values were smaller than those of the corresponding normal groups, and in contrast to the normal findings, the lowest values, 0.2 to 0.4 per cent, occurred in the 4th and 5th week age groups.

Muscle Glycogen.—There was practically no difference in the mean values for muscle glycogen in the youngest groups of diseased and normal rabbits, 0.5 per cent in the 1–3 day interval, and 1.0 per cent in the 7–9 day interval. During the next 4 weeks, somewhat smaller values for both groups were found. From the 25–27 day interval to the 34–36 day interval, the normal values ranged from 0.75 to 0.5 per cent while those for the groups of osteopetrosis rabbits continued at the 0.25 per cent level.

DISCUSSION

The hereditary lethal disease of the rabbit described in this and other papers (1, 2) is characterized by a marked abnormality of the entire skeleton, well demonstrated in x-ray photographs. At birth and for the first few days of life, all bone shadows are homogeneously opaque and lack all finer structural detail. In the 2nd and 3rd weeks of age, less dense areas in the central portions of the bone shadows are seen and with increasing age, these areas become larger and

more translucent. The cortical portions, however, continue to be thickened and the complete differentiation of all marrow cavities has not been observed.

Other evidence of the profound disturbance of bone growth and development is shown by the reduced size of the bone shadows generally and the abnormal shape or outline of some of them. These features are most conspicuous in the long bones of the extremities, particularly the femur. X-ray photographs of cases taken within a few hours of birth indicate that bone growth is slightly subnormal or retarded and in older cases, the bones have a marked stunted appearance. Furthermore, the larger long bones frequently show a bulbous expansion of the growing ends and a greater or less constriction of the central portion of the shaft. A similar clubbing of rib ends is also seen.

There are considerable variations in the degree of differentiation of the bone shadows in any particular case.

In general, the bones of the feet and the sternal and vertebral bones show the greatest amount and the bones of the foreleg more than those of the hindleg; the pelvic bones and the femur show the least. There is often an individual variation in the x-ray appearance of the same bone in different cases of the same or approximately the same age. The individual differences in the retardation of bone growth and in the degree of structural differentiation can in most cases be directly related to the general character and progress of the disease. Less pronounced growth retardation and a greater degree of bone differentiation were usually found in those cases in which the course of the disease was protracted and the marked signs of cachexia developed comparatively slowly. In these cases there was generally a stabilization of body weight, or only a comparatively minor weight loss, over a period of several days just preceding the final fatal outcome.

The close resemblance of the x-ray photographs of the skeleton in this hereditary disease of the rabbit to those of human osteopetrosis or Albers-Schönberg disease strongly suggests an identical condition. In both, the entire skeleton is involved and in a similar manner, that is, the shadows show a homogeneous opacity involving all the cortical and medullary portions of the bone. In the human disease variations from the typical pattern have been reported in the benign type usually seen in adults (11) and also, and perhaps more frequently, in the so called malignant or severe form characteristic of early life (12). These variations comprise areas of lessened density of certain bones or an individual bone appears less opaque than others.

Ellis (13) called attention to the marked similarity of the clinical and x-ray findings in two brothers aged 2 years, 10 months, and 1 year, 6 months. In both cases the main area of sclerosis occurred in the middle third of the shaft of the long bones and outside the area of clubbing. Ellis suggested that different family groups present variations of the condition which are, however, constant within a group.

In the disease of the rabbit, as has just been pointed out, variations in density regularly occur in older cases. In spite of disease progression to an invariably fatal termination generally within 5 weeks of age, the central portions of the bones become much less opaque suggesting a greater or less degree of differentiation of marrow cavities. A similar change is not a typical feature of the x-ray photographs of human cases but it is possible that it is analogous to the transverse and longitudinal bands of greater and lesser density which are described as characteristic findings in the human disease (12, 14, 15). Transverse bands running through the metaphysis of long bones parallel to the epiphyseal line are frequently seen; more rarely longitudinal lines are found in the diaphysis parallel to the periosteum. The presence of the bands has been explained on the basis of periodic remissions and recrudescences of the pathologic deposition of bone and by periodic recurrences of widespread resorption (16). This difference in the x-ray appearance of the bones may be related to differences in the rate of growth and of disease progression in the two species.

Other skeletal changes in human osteopetrosis and especially the severe form, have their counterpart in the disease of the rabbit. Retarded growth in bone length and diameter is described while expansion and clubbing of the ends of the bones, particularly the femur, is a characteristic feature. On the other hand, spontaneous fractures which occur fairly frequently in human cases have not been observed in the rabbit. At postmortem examination the bones are described as more fragile or brittle than normal and this was also found in the rabbit (2).

The complex of hereditary osteopetrosis of the rabbit further includes well marked hematologic abnormalities. An anemia, usually macrocytic in type, was generally observed and in the longer lived cases it was frequently severe. At about 3 weeks of age, signs of retarded development and malnutrition are well established (1) and with disease progression, lower red cell counts and lower hemoglobin and hematocrit readings were typical findings.

In the age period of 22 to 39 days, there were 36 counts on osteopetrosis cases and 55 on normal litter mates (Chart 1) and for the present general comparison, the results may be combined and expressed in terms of mean values. For the osteopetrosis and the normal groups the mean values were: erythrocytes 3,290,000 and 4,390,000 per c.mm. respectively; hemoglobin 7.28 and 8.13 gm. per 100 cc. respectively; and hematocrit readings of 26 and 32 per cent respectively. The mean body weight values were 235 gm. for the osteopetrosis and 519 gm. for the normal rabbits.

Other typical hematologic findings included increased values for mean corpuscular volume, slightly higher mean corpuscular hemoglobin content and mean corpuscular hemoglobin concentration as compared with the levels of normal litter mates. There were also increased reticulocyte counts, a con-

tinued presence of normoblasts, and a persistence of polychromatophilia and basophilic stippling of the erythrocytes. The mean platelet counts were consistently lower than those of the normal litter mates.

A moderate leucocytosis was usually present and as a rule was most pronounced in advanced cases, 3 or 4 weeks of age and older.

The mean white counts of the most advanced cases examined in the 6th week of age were approximately 75 per cent larger than the mean values of the youngest cases examined in the 1-3 day age group. They also exceeded corresponding mean normal values. During the first 3 weeks of life, the relative proportions of granular and non-granular leucocytes in the osteopetrosis rabbits conformed to a normal distribution but thereafter the proportion of granular cells became increasingly higher and the lymphocyte values correspondingly lower.

Of special interest was the occurrence of immature polymorphonuclear leucocytes in practically every case together with a high case incidence of myelocytes. Additional evidence of marked hematologic disturbance included basophilic toxic degeneration of neutrophils and lymphocytes in about one-fifth of the cases, including many very young cases. Vacuolization of neutrophils and lymphocytes and ruptured leucocytes were also seen.

The similarity of these hematologic findings to those observed in many human cases of osteopetrosis is very striking. Most of the cases occur in children under 10 years of age and the majority have an anemia which is frequently described as a myelophthitic anemia and other hematologic abnormalities as well. Vaughan (17) states that while a few instances of hypochromic anemia are recorded, the usual picture is that of a leucoerythroblastic anemia; a few immature myeloid cells and all types of nucleated red cells are found in stained films. The relationship between the hemopoietic condition and the signs and symptoms and the progressive course of the disease has been emphasized by many authors.

Rosenthal and Erf (18) for example, point out that the characteristic symptoms of the severe form of osteopetrosis seen in infants and children include progressive development of a refractory type of anemia associated usually with thrombopenia or leucopenia and often with slight myelemia. Lamb and Jackson (19) attribute the gravest symptoms to the associated hemopoietic disturbances which are accompanied by severe anemia and leucemic states. Severe hemorrhages, which were not a feature of the rabbit disease, have been reported in human cases (20, 21).

In the so called benign form of osteopetrosis seen in adults the blood picture is usually essentially normal. In certain older cases, however, the disease may be more severe and hematologic disturbances have been reported, as in Mettier's (22) patient aged 50 years.

The chemical observations on rabbits with osteopetrosis revealed several interesting differences from normal values obtained from litter mates of the

same age and in certain respects they are comparable to the scanty available data from human cases. The results expressed in terms of mean group values at 3 day intervals from 1 to 36 days of age showed first, that the serum calcium values of the diseased animals were consistently lower than those of the normal rabbits and second, that there was a progressive drop in these values which was particularly marked by the 4th week of age. After about 10 days of age the calcium levels of the normal groups were comparatively constant around 16 mg. per 100 cc. serum. The lowest values for the diseased groups in the 4th and 5th weeks were approximately half those of the corresponding normal groups.

The mean serum phosphorus values of the osteopetrosis rabbits were much smaller than those of the normal animals during the first 4 weeks of age but were somewhat higher in the 5th week. The levels of the normal groups ranged from 8 to 9 mg. per 100 cc. serum in the 1st month while in the 5th week, the slightly lower level of 7.5 mg. was well maintained. For the youngest osteopetrosis groups mean values of 5 to 6 mg. were found but beginning at about 2 weeks of age, the values became increasingly higher and in the 5th week, a level of approximately 8.5 mg. per 100 cc. serum prevailed.

The mean calcium:phosphorus ratios for the groups of normal rabbits varied around 2.0. The ratios for the osteopetrosis groups showed an irregular but progressive decline from the high values of 3.0 and 3.2 for the youngest groups to levels of 0.8 for the groups aged 25 to 30 days. After 3 weeks of age, all the mean ratios were smaller than those of the normal groups.

In human osteopetrosis, the serum calcium and phosphorus have usually been reported as normal (23) or somewhat reduced (14) and values in the latter category appear to be characteristic of the severe form of the disease.

Robertson's (21) case, for example, at 10 months of age had 10.1 mg. calcium and 4.0 mg. phosphorus per 100 cc. serum; at 23 months the values were 9.9 and 2.9 mg. respectively. Other instances of lower range value in cases in infants and young children have been reported by Kramer and Halpert (24), van Creveld and Heybroek (25), and Ellis (13).

All the mean serum phosphatase values of the groups of rabbits with osteopetrosis exceeded those of the normal litter mates and the differences were especially marked in the younger animals up to about 3 weeks of age. In the 10-12 day interval, for example, the mean phosphatase values were 14.5 Bodansky units for the osteopetrosis and 8.2 units for the normal group per 100 cc. serum; in the groups in the 4th and 5th weeks of age, the general level was 10 units for the osteopetrosis and 7 units for the normal groups per 100 cc. serum.

Only a few reports on cases of human osteopetrosis refer to determinations of serum phosphatase and although the results are variable there is a suggestion of increased values, particularly in young patients.

In Bodansky and Jaffe's (23) patient aged 16 years, values of 15.5 to 21.8 Bodansky units were found, that is, 2 to 3 times the average value of that found in a child and about 5 to 8 times that of an adult. An increased level of 23 Bodansky units was found in a fatal case aged 3½ months (24). In certain other cases, the values tended to be high (13, 17, 20) but lower values have also been reported (13, 25).

The mean sugar content of the blood in rabbits with osteopetrosis was much lower than in normal litter mates in all age groups examined except those in the 1st week of life in which no difference was found. From the few available determinations in human cases, the blood sugar appears to be in the lower range of normal values (18, 20, 26).

In the case of blood cholesterol, the results for both classes of rabbits were irregular but all mean values for the groups of diseased rabbits were higher than those for the normal litter mates. The results in human cases suggest normal or increased blood cholesterol values (13, 20, 25, 26, 27).

All mean values for liver glycogen in the osteopetrosis rabbits were smaller than those of the corresponding groups of normal litter mates and the difference was greatest in the older groups. In the case of muscle glycogen the results indicate that somewhat smaller values occur in osteopetrosis than in normal rabbits. No reports on liver and muscle glycogen determinations in human cases are available.

The hematologic and blood chemical changes found in rabbits with osteopetrosis emphasize the serious nature of the disease and their progressive character roughly parallels the retardation of growth and the deterioration in the general condition of the animal which regularly develop. The abnormal blood picture and to some extent the blood chemical findings, the homogeneous dense x-ray appearance of the entire skeleton present at birth, underdevelopment and retarded growth, the comparatively early lethal outcome, and an hereditary basis are typical features of this disease which also characterize the severe juvenile form of osteopetrosis of man. The similarity of the pathologic findings will be shown in a following paper (2).

SUMMARY

The results of x-ray, hematologic, and chemical studies on cases of hereditary osteopetrosis of the rabbit are described and the resemblance of the findings to those of the severe juvenile form of human osteopetrosis is pointed out.

The outstanding feature of the x-ray examinations was the homogeneous dense appearance of the entire skeleton. This condition was present at birth. In older cases there was evidence of some differentiation of bone structure.

The hematologic studies showed that the disease was characterized by the development of a macrocytic anemia, thrombocytopenia, and a moderate myeloid leucocytosis. Other abnormal findings included high reticulocyte and

normoblast counts, anisocytosis and poikilocytosis, and degenerative changes of the neutrophils and lymphocytes.

The chemical studies showed very low serum calcium values; serum phosphorus values were low during the first 4 weeks of life but were somewhat higher than normal levels in older cases. The serum phosphatase values were elevated. The blood sugar content was generally low. The blood cholesterol values were generally high. The liver glycogen values were small especially in older cases and those for muscle glycogen were somewhat smaller than normal values.

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EXPLANATION OF PLATES

All the rabbits were killed with chloroform except the one providing Fig. 8. In the x-ray photographs the dorsal surface of the body or of the dissected skeleton was in contact with the film holder. In Figs. 3 to 7, 9, 13 to 16, and 19 and 20 certain parts of the skeleton, such as the cranial vault, the sternum, the base of the skull and the mandible are shown separated from the main specimen.

PLATE 36

FIGS. 1 and 2. X-ray photographs of the skeleton of a case of osteopetrosis of a new-born rabbit and of a normal litter mate. Both were males and weighed 43 and 50 gm. respectively. The stage of bone development of the osteopetrosis rabbit (Fig. 1) is slightly less advanced than that of the normal sib (Fig. 2) as is indicated particularly by the difference in the epiphyseal shadows. In the bones of the extremities there are practically no marrow cavities; in the vertebrae, ribs, and pelvis the marrow cavities are ill defined and small and are much less extensive than those in the normal litter mate. Calcification of the calvarium is deficient. The lower incisor teeth were erupted; the upper incisors and the upper and lower molars were not erupted. $\times 0.54$.

FIGS. 3 and 4. X-ray photographs of the skeleton of an osteopetrosis rabbit and of a normal litter mate aged 4 days. Both were males and weighed 90 and 140 gm. respectively. The general condition of the diseased rabbit was excellent but the growth rate was already slower than that of the normal sib. At birth, the osteopetrosis rabbit weighed 55 and the normal 70 gm. respectively; in 4 days the weight of the abnormal animal had increased by 64 per cent as compared with 100 per cent for the normal. The shadows of the long bones, sternum, and pelvis of the rabbit with osteopetrosis (Fig. 3) are of uniform density except for very small central areas of slightly lessened intense shadow. There is a suggestion of slightly more marrow space in the ribs, the bodies of the vertebrae, and probably in the bones of the base of the skull and in the scapula. The upper incisor teeth and the right lower incisor were tiny barely palpable points while the left lower was a small twisted tooth tilted inward toward the midline. $\times 0.54$.



1



2



3



4

PLATE 37

FIGS. 5 and 6. X-ray photographs of the skeleton of an osteopetrosis rabbit and of a normal litter mate 9 days old. Both were females and weighed 154 and 200 gm. respectively. The diseased rabbit was in good condition and was gaining weight. As shown in Fig. 5 the bones of the extremities and pelvis are represented by dense, practically uniform shadows. In other bones, particularly the vertebrae, ribs, and sternum there are small central areas of less intense density. None of the incisor teeth was erupted and none can be seen in the x-ray photograph. The outline of only 2 small molar teeth in each mandible can be seen as compared with 3 in the normal animal; in the maxilla of both animals, 8 molars can be identified but those of the osteopetrosis rabbit are smaller. In general, the skeleton of this animal is smaller than that of the normal sib, but the difference is not as marked as usual. A photograph showing the toothless condition is depicted in the preceding paper (1, Fig. 3). $\times 0.54$.

FIG. 7. X-ray photograph of the skeleton of an osteopetrosis male rabbit 13 days old and weighing 132 gm. The nutritional state was good. Note that there is no indication of a marrow cavity in the pelvic and femoral bones and only a suggestion of it in the slightly less dense shadows of the central portions of the other long bones. In the ribs, sternum, and vertebrae, the marrow cavities are somewhat more distinctly indicated. The long bones and especially the femur and tibia show shortening. Calcification of the skull and mandible is subnormal. The left upper incisor was not erupted and only the point of the right was visible; the lower incisors were separated but were of normal length. The upper molars were small somewhat irregular teeth while the lower molars were just erupting. $\times 0.54$.

FIG. 8. X-ray photograph of the skeleton of an osteopetrosis female rabbit 15 days old and weighing 95 gm. It was found drowned in the water trough; there was very little post-mortem decomposition. The growth of this animal had been extremely limited. The birth weight of 45 gm. had only doubled in a fortnight, whereas that of normal animals of this stock increases three- or fourfold or even as much as six times in this period (1, Charts 3 and 4). The pronounced shortening and peculiar shape of the femoral bones should be noted. Calcification of the vault of the skull was deficient, the posterior fontanelle was not closed, and hydrocephalus was present. The fracture of the right humerus was an autopsy accident and the bones generally were brittle. Only tiny points of the upper incisor teeth could be seen; the left lower incisor was missing and the right lower was a very thin slanting tooth. The molar teeth were subnormal. $\times 0.54$.



PLATE 38

FIG. 9. X-ray photograph of the skeleton of an osteopetrosis female rabbit aged 13 days. The animal was still in comparatively good physical condition and weighed 140 gm. but there had been no gain in weight since the 9th day. The body weight values of this rabbit and 3 normal sibs are portrayed by a series of curves in the preceding paper (1, Chart 4); curve D represents the values for this rabbit. A photograph of the incisor teeth is also shown (1, Fig. 4). The bone shadows are typical of the condition. An unusual feature is the indentation of the 8th and 9th right ribs (to the observer's left) which is shown more clearly in Fig. 10. $\times 0.54$.

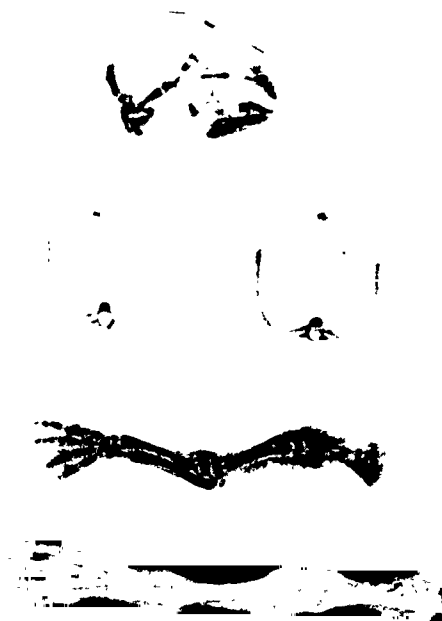
FIG. 10. X-ray photograph of the anterior or ventral portion of the ribs of the osteopetrosis animal shown in Fig. 9 and, below, a similar region of a normal animal of the same age and belonging to the same stock. The expansion of the ventral ends of the ribs together with an intensification of shadow is frequently seen in the x-rays of osteopetrosis rabbits of this age. $\times 0.54$.

FIG. 11. X-ray photographs of bones of an osteopetrosis female rabbit 15 days old and weighing 198 gm. The physical condition was good and the body weight was still increasing. The deficient calcification of the calvarium and of the 9th and 10th ribs and vertebrae are well shown. The 9th ribs and vertebra of a normal litter mate appear in Fig. 12. In the central portion of the long bones the shadows are not uniformly dense but show irregular and spotty areas of lessened density. None of the incisor teeth could be seen or felt. The posterior calvarium was prominent but hydrocephalus was not found. The bones of another osteopetrosis sib and a normal rabbit of this litter and of the same age are shown in Fig. 12. $\times 0.54$.

FIG. 12. X-ray photographs of the bones of an upper extremity of an osteopetrosis rabbit to the left and of a normal sib to the right, aged 15 days. Below are the 9th ribs and vertebra of the normal rabbit. These animals were litter mates of the rabbit of Fig. 11. Both were males. The normal animal weighed 550 gm. The osteopetrosis animal weighed 276 gm., was in quite good condition and was still growing. It will be noted that the marrow cavities of the long bones were fairly well defined and more so than those of the other osteopetrosis sib (Fig. 11). $\times 0.54$.



10



11



12

PLATE 39

FIG. 13. X-ray photograph of the skeleton of an osteopetrosis male rabbit aged 21 days and weighing 150 gm. The dense shadows of the bones of the extremities and of the pelvic bones are particularly prominent and there are only faint indications of marrow spaces. The ventral ends of some of the ribs are beaded.

This rabbit was the smallest of a litter of 5 which included another case of osteopetrosis; the birth weight was 35 gm. as compared with 41 to 46 gm. for the others. Growth had been steady until the last 4 days when there was only a slight increase of weight but the general condition was still fairly good. At 15 days of age, hydrocephalus was strongly suspected; the posterior fontanelle had not completely closed. There was no evidence of hydrocephalus, however, at autopsy 6 days later. The upper incisors were tiny infantile teeth which had grown but little and the left lower was smaller than the right lower. $\times 0.54$.

FIG. 14. X-ray photograph of bones of an osteopetrosis male rabbit aged 31 days and weighing 340 gm. This animal was an example of the relatively infrequent case which is still growing at this age and which has shown little or no cachexia. The comparatively slow process of the disease is reflected in the appearance of the bones. In the femur and pelvis the marrow cavity is still not well defined but the appearance of the other bones is less abnormal. The condition of the teeth, however, was characteristic. The right upper and the left lower incisor teeth were long, frail, and spindly while the left upper and right lower incisors were only very tiny points. At birth, all the incisors were erupted but the uppers were shorter than normal. At 3 days, the left upper and the right lower were very small and evidently not growing. On the 10th day, the left upper had been shed, the right upper was a tiny structure, and the lowers were normal looking. During the next fortnight, a second left upper incisor erupted but grew very little while the left lower developed into a long frail tooth. In the following week, the right upper became long and spindly. It is probable that the tiny right lower incisor noted on the 31st day when the animal was killed was a second tooth although the shedding of the first tooth was not observed. $\times 0.54$.

FIGS. 15 and 16. X-ray photographs of bones of an osteopetrosis tarda rabbit and of a normal litter mate aged 24 days. Both were males. The normal animal (Fig. 16) weighed 324 gm. The osteopetrosis sib weighed 156 gm.; the cachectic condition was progressing quite rapidly, there was a loss of weight, and diarrhea had developed. The typical density of osteopetrosis bones is well shown. In the femur, tibia, and pelvic bones, there are only slight indications of marrow spaces and the degree of shortening of the long bones is marked. At birth all the incisor teeth were normal but by the end of the 1st week, the uppers had grown only slightly. On the 10th day, the right lower was missing and the left lower was a spindly frail tooth. When the animal was killed, the upper incisors were somewhat short, especially the right, and both slanted toward the right side of the body. A second right lower incisor (to the observer's left) had erupted and was placed partly behind the larger left lower tooth. Hydrocephalus was thought to have developed at 10 days and to have persisted for at least a week by which time the posterior fontanelle had closed. The posterior calvarium was still prominent at 24 days of age but no evidence of hydrocephalus was found at autopsy. $\times 0.54$.



PLATE 40

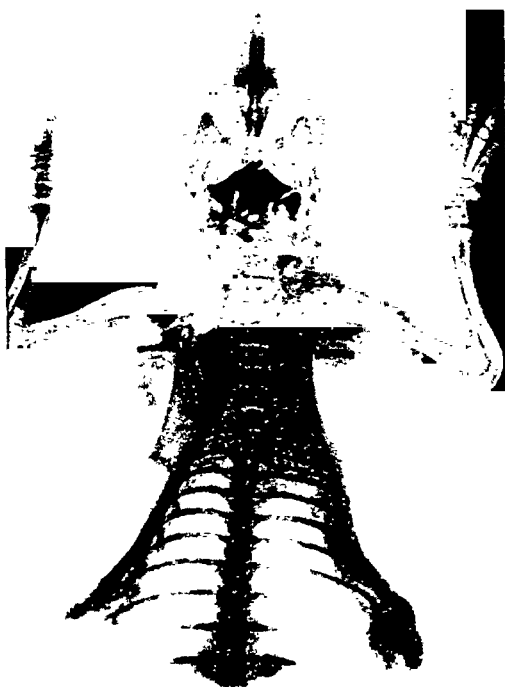
FIGS. 17 and 18. X-ray photographs of the anterior skeleton of an osteopetrosis rabbit and a normal litter mate aged 27 days. Both were males and weighed 308 and 680 gm. respectively. The osteopetrosis animal was still well nourished but had been losing weight for the preceding 4 days. The comparatively small size of the bones generally and the shortened long bones are well shown. The general appearance and the extent of many marrow spaces approach normal but in others and especially in the humerus, there is still a considerable degree of spotty irregular clouding. The cortex of the humerus, radius, and ulna is thickened. The ribs appear to be somewhat wider than those of the normal litter mate and the vertebral ends are expanded. The rib shadows are not particularly dense; in general they are homogeneous and lack the cortical lines of more intense shadow seen in the normal animal. The incisor teeth were markedly abnormal. The right upper was only a tiny point and the left was missing; the lowers were widely separated and the left lower was smaller and shorter than the right. A photograph of the teeth on the 16th day is reproduced in the preceding paper (1, Fig. 5), together with photographs of both rabbits on the 16th and 24th days (1, Figs. 11 and 12). $\times 0.54$.

FIGS. 19 and 20. X-ray photograph of bones of an osteopetrosis rabbit and a normal litter mate aged 34 days. Both were males and weighed 230 and 646 gm. respectively. The osteopetrosis rabbit was almost moribund. At birth, the weight was 52 gm.; for about a fortnight, the general condition was excellent, active growth took place, and on the 14th day, the body weight was 200 gm. The comparable weights for the normal litter mate were 30 and 220 gm. respectively. During the 3rd week the condition of the osteopetrosis rabbit underwent a marked deterioration, a progressive malnutrition developed, and a retardation and then cessation of growth were observed. At the end of the 4th week, diarrhea developed. Within a day or two the weakness was obviously intensified and by the 34th day, the animal was prostrated.

The contrast in the x-ray photographs of these litter mate rabbits is very striking. In the rabbit with osteopetrosis (Fig. 19) the bones generally are very much smaller than those of the normal sib (Fig. 20). The characteristic shortening and thickening of the long bones is particularly well brought out in the case of the femur and tibia. In both these bones the marrow cavities are still not well differentiated; in the pelvis, vertebrae, and base of the skull, there is a similar although less pronounced condition. In the sternal, tarsal, metatarsal, and phalangeal bones the marrow spaces are better differentiated. In the x-ray film of the upper part of the skeleton not reproduced here the ribs resemble those shown in Fig. 17 with the additional feature of a considerable bulbous expansion of their anterior or ventral ends. The right upper incisor tooth was missing and the left upper was merely a tiny point. Both secondary incisors were also very small points. The lower incisors were separated, long teeth, the right longer than the left. The molar teeth were small and irregular. As shown in the x-ray photograph only the 2 posterior upper molars were developed to a degree approaching normal. $\times 0.54$.



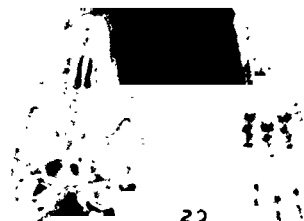
17



18



19



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THE ASSOCIATIVE REACTIONS OF PNEUMONIA VIRUS OF MICE (PVM) AND INFLUENZA VIRUSES: THE EFFECTS OF pH AND ELECTROLYTES UPON VIRUS-HOST CELL COMBINATIONS

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Previous studies (1-4) on pneumonia virus of mice (PVM) have shown that the virus combines either with erythrocytes or with lung particles obtained from species susceptible to infection with the agent but not with other tissues. Moreover, it has been demonstrated (1-5) that, unlike influenza (6), mumps (7), and Newcastle disease (8) viruses, spontaneous dissociation from the combined state does not occur with PVM. Release of the virus from combination can be achieved by heat (1-3) or treatment with alkali (5) but either procedure destroys the infectivity of the virus, as well as the combining capacity of the tissue component, although neither demonstrably alters the combining capacity of the virus itself.

In the course of the present investigation a technique was developed by means of which dissociation of PVM from the combined state can be accomplished without destroying either the infectivity of the virus, the combining capacity of the virus, or the combining capacity of the tissue component. This technique provided a means suitable for a study of some of the factors which influence virus-tissue component combinations.

In the present investigation the effects of electrolyte concentration, ionic composition, and pH, as well as virus concentration upon combination have been studied. It will be shown that dissociation of PVM from the combined state is strikingly dependent upon electrolyte concentration and pH; that the effect of electrolytes upon combination between the virus and tissue components is independent of their ionic composition but is dependent upon their concentration; that with respect to combination there is an inverse relationship between virus concentration and electrolyte concentration. In addition, it will be shown that the reactions between influenza viruses and erythrocytes also are strikingly influenced by the ionic environment and certain of the similarities and differences between the reactions of PVM and the influenza viruses with erythrocytes will be presented.

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† A thesis based upon this study was submitted in partial fulfillment of the requirements for the degree of Doctor of Medical Sciences in the Department of Medicine of Columbia University.

Materials and Methods

PVM.—Strain 15 of the pneumonia virus of mice (PVM) (9) was used exclusively. It was maintained by occasional passage in albino Swiss mice and stored as a 10 per cent suspension of infected mouse lungs at -70°C . Either *combined virus* or *heat-released virus* suspensions were prepared as described in previous communications (5, 10). *Dissociated PVM* preparations were obtained from 10 per cent suspensions of perfused infected lungs in non-electrolyte solutions (i.e., distilled H_2O , 0.25 M dextrose, or 0.25 M sucrose) and were centrifuged at 12,000 R.P.M. for 30 minutes in order to sediment the tissue-combining component (4).

Influenza Viruses.—The PR8 and FM1 strains of influenza A virus, and the Lee and B1103¹ strains of influenza B virus were employed. The viruses were passed in the allantoic sac of chick embryos according to the technique previously described (11).

Hemagglutination Titrations.—(1) *PVM*: The technique of hemagglutination titrations with mouse RBC, utilizing serial twofold dilutions, and the method of estimating end points were identical with those previously employed (3). (2) *Influenza virus*: The technique of hemagglutination titrations with 1 per cent chicken RBC has been described elsewhere (11).

RBC Suspensions.—Blood was obtained from either mice or chickens and was mixed with 2.5 per cent sodium citrate. The erythrocytes were washed 3 times in the desired diluent, as indicated in the text, and made up by volume to the desired concentration.

Normal Mouse Lung Suspensions.—The lungs of normal Swiss mice were perfused *in situ* with buffered saline. After removal the lungs were ground to a 10 per cent suspension in a modified blender for 2 minutes at 4°C . As indicated in the text, the diluent employed varied in accordance with the experiment. Suspensions were stored at -30°C . until used.

Virus-Combining Capacity of Mouse Lung Suspensions.—As described previously (4) the virus-combining capacity of mouse lung suspensions was taken as the highest dilution which completely combined with either 8 or 16 hemagglutinating units of PVM.

Solutions.—The solutions used frequently in this investigation are designated as follows in the text: Saline = 0.15 M NaCl solution buffered at pH 7.2 with 0.01 M phosphate. Dextrose or sucrose = 0.25 M dextrose or sucrose solution buffered as above. Water = distilled water which was used exclusively.

EXPERIMENTAL

Dissociation of PVM-Lung Particle Combination.—Previous studies have shown that, although PVM cannot be demonstrated by the hemagglutination technique in suspensions of infected mouse lungs unless they are subjected to treatment which releases the virus from combination with tissue particles (1-5), the virus can be so demonstrated with slices of unground lungs (5) as well as in tracheal washings from infected lungs (4). This indicates that in the intact infected lung some virus is present in an uncombined state, and that combination between free PVM and lung particles occurs when the lungs are ground.

In the course of studies on the mechanism of combination between PVM and lung particles, it was observed that as the NaCl concentration of the mixture was reduced the quantity of virus that combined with tissue particles also was reduced. This observation suggested that combination between

¹ The B1103 strain was obtained through the courtesy of Dr. R. M. Taylor, the International Health Division, The Rockefeller Foundation, New York, N. Y.

PVM and lung particles could be prevented, or that such combination once formed could be dissociated, by grinding infected lungs either in water or in a non-electrolyte solution so that the electrolyte concentration of the suspension would be low. To test this hypothesis a number of different experiments were carried out.

The lungs of mice infected with PVM were perfused with saline to remove both erythrocytes and antibodies against the virus which, if allowed to combine with PVM during grinding, would reduce the amount of virus obtained. Ten per cent suspensions then were made in either saline, water, 0.25 M dextrose, or 0.25 M sucrose. Aliquots of each of the suspensions were heated at 70°C. for 30 minutes and clarified by centrifugation. Additional aliquots were

TABLE I

The Dissociation of PVM from Infected Mouse Lung Suspensions in Distilled H₂O or Solutions of Non-Electrolytes

PVM suspension: 10 per cent perfused infected mouse lung prepared in	Treatment of suspension		Hemagglutination titer of supernate vs. mouse RBC
	70°C.	12,000 R.P.M.	
	min.	min.	
0.15 M NaCl	0	0	0
" " "	30	10	256
Distilled H ₂ O	0	30	256
" " "	30	10	1024
0.25 M dextrose	0	30	512
" " "	30	10	2048
0.25 M sucrose	0	30	512
" " "	30	10	2048

immediately centrifuged. Each aliquot then was tested by the hemagglutination technique and the titer of free virus was determined.

The results of certain typical experiments are shown in Table I. As has been recorded previously (1-3) no virus was detectable by hemagglutination with unheated PVM suspensions prepared in saline but the agent was demonstrable when such suspensions were heated appropriately. On the other hand, virus was readily demonstrated by hemagglutination with suspensions prepared in either water, dextrose, or sucrose. Suspensions of normal mouse lungs, prepared in identical manners, did not cause hemagglutination reactions similar to those obtained with infected lungs. In some instances, with low dilutions of normal lung suspensions, slight aggregation or clumping of erythrocytes was observed; both qualitatively and quantitatively such clumping was readily distinguishable from that caused by the virus. Moreover, specific

serological tests (3) clearly demonstrated that the hemagglutination reactions obtained with suspensions of infected lungs, containing but little electrolyte, were attributable to PVM. A suspension of the virus in 0.25 M sucrose, which gave a hemagglutination titer of 1:512, caused no hemagglutination after it was mixed with PVM immune hamster serum.

The results of these experiments suggest that when the electrolyte concentration of the suspension is maintained at a low level PVM either does not combine with or dissociates from tissue particles formed during grinding of mouse lungs. With the techniques now available it is impossible to determine how much of the virus in intact infected lungs is combined and how much is uncombined. Nor is it possible to determine directly whether virus first combines with and then dissociates from particles formed when infected lungs are ground in non-electrolyte solutions, or whether such combination is prevented. However, when combination between the virus and lung particles is caused to occur by grinding infected lungs in saline, the combined virus can be dissociated readily by reducing the electrolyte concentration to a low level. A distinction between the alternatives, therefore, would appear to be unimportant and the term *dissociated virus* will be used to designate the uncombined virus present in the supernate of preparations ground in solutions of low electrolyte concentration.

Table I shows, in addition, that the hemagglutination titer of virus obtained with preparations of dissociated PVM is usually equal to or greater than the titer obtained with heat-released virus in saline. However, the titer of suspensions heated in non-electrolyte solutions is in general fourfold greater than the titer obtained with either heat-released virus in saline or dissociated virus. It is evident that the hemagglutination titer of heat-released virus in saline, or dissociated virus in non-electrolyte solutions, reveals but a small proportion (*i.e.*, of the order of 25 per cent) of the virus present in infected mouse lungs as determined by hemagglutination with suspensions heated in non-electrolyte solutions.

Infectivity of Dissociated PVM.—Either heat or alkali treatment, procedures used previously to release PVM from combination in infected lung suspensions, renders the virus non-infectious (1-5). On the other hand, *free infectious PVM*, prepared by extracting unground lung slices with cold saline, has been shown to be infective (5). It was important to determine if dissociated virus preparations also were infectious.

Tests for infectivity were carried out in the following manner. A suspension of dissociated PVM was prepared in 0.25 M dextrose. In order not to cause inactivation of the very unstable property of infectivity, the dextrose solution was buffered at pH 7.2 with 0.01 M phosphate, and centrifugation was carried out at 4°C. The supernate was diluted in cold buffered dextrose and the virus titered in mice as described elsewhere (5).

It was found that the dissociated PVM preparation employed, which had a hemagglutination titer of $10^{-3.1}$, had an M.S.50 virus titration end point of $10^{-2.2}$. The latter figure is comparable to the infectivity titers obtained previously with free infectious virus of similar hemagglutination titer (5). It is evident that following dissociation in the presence of dextrose PVM retained the property of infectivity.

The Effect of Centrifugation on Dissociated PVM.—The effects of centrifugation and of heat upon dissociated virus were studied in order to provide a basis for the interpretation of experiments reported in subsequent sections.

TABLE II
Effect of Centrifugation on Dissociated PVM in Distilled H₂O

PVM suspension: 10 per cent perfused infected mouse lung prepared in	Treatment of suspension				Hemagglutina- tion titer of supernate vs. mouse RBC
	NaCl added to	70°C.	Centrifugation		
			<i>M</i>	<i>min.</i>	
Distilled H ₂ O	0	0	12,000*	30	256
" "	"	"	"	60	512
" "	"	"	18,000†	166	32
" "	"	30	8,000	5	512
" "	"	"	12,000	30	"
" "	0.15	"	8,000	5	256
" "	"	"	12,000	30	"
0.15 M NaCl	0	"	8,000	5	128
" " "	"	"	12,000	30	"

* Rotor diameter = 19.8 cm.

† Rotor diameter = 30.1 cm.

A suspension of infected lungs in water was divided into aliquots which were centrifuged at high speed under various conditions. In certain instances the specimens were heated at 70°C. for 30 minutes; in others NaCl to 0.15 M was added before heating; in still others neither the addition of NaCl nor heating was employed. A suspension of heat-released virus in saline served as a control preparation. The hemagglutination titer of the supernate from each aliquot was determined after it had been centrifuged at 8,000, 12,000, or 18,000 R.P.M. for various periods. When the latter speed was desired, a high speed vacuum centrifuge (12) with a rotor about 1.5 times the diameter (i.e., 30.1 cm.) of that of the open air high speed centrifuge (13) was employed. The greatest centrifugal force used was, on the basis of previous studies on the sedimentation of uncombined PVM (10), sufficient to sediment approximately 90 per cent of the free virus.

The results of these experiments are presented in Table II. It will be seen that, as was found previously (10), the centrifugal force used did not cause sedimentation of a significant amount of heat-released virus. Similarly, it

was found that PVM, dissociated from combination in water, showed no increased sedimentation in high gravitational fields and, moreover, was unaffected by heating in the presence or absence of added NaCl. Finally, approximately 90 per cent of the dissociated virus was sedimented by a gravitational force calculated to sediment the uncombined virus. These results indicate that the procedures employed did not lead to aggregation of the virus particles and suggest that dissociated PVM is not significantly larger than either heat-released virus or free infectious virus obtained from intact lungs (10). The effect of heating dissociated virus was also studied. It was found that dissociated virus showed heat stability similar to that of combined PVM.

The Effect of Centrifugation and Heat on Lung Particles in Water.—For reasons mentioned in the preceding section, studies were made on the effect of centrifugation and of heat on tissue particles present in suspensions of normal mouse lungs in either saline or water.

Suspensions in these diluents were centrifuged at 12,000 R.P.M. for 15, 30, and 60 minutes, respectively, and the virus-combining capacity of the supernates was determined in the presence of 0.15 M NaCl as described above.

No significant difference in the combining capacity of the water and saline supernates was observed. These results indicate that grinding mouse lungs in water does not yield tissue particles which are less readily sedimented than those present in lungs ground in saline.

Studies on the heat stability of lung particles in saline and in water were carried out. Suspensions of normal mouse lungs in these diluents were heated at 70°C. for 30 minutes and their virus-combining capacity then was determined. It was found with both types of suspension that the capacity to combine with PVM was destroyed by this treatment. These results indicate that the combining capacity of particles suspended in water is not more heat-stable than that of lung particles in saline.

Dialysis of Dissociated PVM.—In the preceding experiment with dissociated PVM, although virus suspensions were prepared in water or in solutions of non-electrolytes, they still contained the electrolytes present in the infected lung tissue itself. In order to remove the diffusible portion of such electrolytes, suspensions of infected lungs in water were dialyzed in cellophane against a large volume of water for 24 hours at 4°C. The properties of dialyzed suspensions of dissociated PVM were then investigated.

It was found that a greater proportion (*i.e.*, of the order of 50 per cent) of the virus present in dialyzed suspensions was demonstrable by hemagglutination than in undialyzed suspensions prepared in water. Combination and dissociation between dialyzed virus and dialyzed lung particles, obtained either from infected or normal lungs, can be caused to occur at will by appropriate manipulation of the NaCl concentration of the mixture as is indi-

cated below. The increased sedimentation of heat-released PVM following dialysis (3) also was observed to occur with dialyzed dissociated PVM. An unexpected finding was the observation that dialyzed suspensions lost the greater proportion of their hemagglutinating capacity if heated in the absence of salt. This property remained unaffected if NaCl were added to the dialyzed suspension prior to heating.

Recombination of Dissociated PVM and Lung Particles.—The finding that dissociation of PVM–lung particle complexes can be accomplished by lowering the electrolyte concentration raised questions as to whether combination and subsequent dissociation had altered the combining capacity of either the virus or the tissue particles, and whether recombination could be caused to occur

TABLE III

Recombination between Dissociated PVM and Mouse Lung Particles in the Presence of NaCl

PVM suspension: 10 per cent perfused infected mouse lung prepared in	Treatment of suspension			Treatment of supernate		Hemaggluti- nation titer of supernate rr. mouse RBC
	70°C.	NaCl added	12,000 R.P.M.	Normal mouse lung particles added in	12,000 R.P.M.	
	<i>min.</i>	<i>M</i>	<i>min.</i>		<i>min.</i>	
Distilled H ₂ O	0	0	30	0	0	128
" "	"	0.15	"	"	"	0
" "	30	0	10	"	"	128
" "	"	"	"	Distilled H ₂ O	30	"
0.15 M NaCl	"	"	"	0	0	"
" " "	"	"	"	0.15 M NaCl	30	0

by raising the electrolyte concentration of the mixture. The following experiments were carried out.

A suspension of infected lungs in water was divided into aliquots. One was centrifuged in the usual manner. To the other was added NaCl to 0.15 M, after which it was centrifuged similarly. To a heat-released PVM preparation in water was added an equal volume of a suspension of normal mouse lungs in water. The mixture was held at 37°C. for 30 minutes and then centrifuged to remove the lung particles. As a control, a heat-released PVM suspension in saline was employed. To one volume was added an equal volume of a suspension of normal lungs in saline. The mixture was incubated and then centrifuged as above. Each of the supernates was tested for the presence of PVM by the hemagglutination technique.

The results of these experiments are presented in Table III. As has been demonstrated previously (2, 3), heat-released virus combined with lung particles in saline and was not demonstrable by hemagglutination. It is evident that in low concentrations of NaCl dissociated PVM did not recombine with lung particles to an appreciable extent. However, when NaCl was added in sufficient concentration dissociated virus and lung particles did recombine.

This demonstrates clearly that the combining capacity of neither the virus nor lung particles is irreversibly altered after dissociation has been effected.

Effect of NaCl Concentration upon Recombination between Dissociated PVM and Lung Particles.—The finding that dissociated PVM recombined with lung particles when NaCl was added suggested that the reaction could be used to determine the relationship between NaCl concentration and recombination of the virus and lung particles.

To aliquots of a suspension of infected lungs in water NaCl was added to yield concentrations ranging from 0.145 to 0.018 M. The mixtures were held at room temperature for 20 minutes, centrifuged at 12,000 R.P.M. for 30 minutes, and the supernates tested by the hemagglutination technique.

The results of this experiment are shown in Table IV. Under the experimental conditions employed, recombination between dissociated PVM and

TABLE IV

The Effect of NaCl Concentration on Recombination between Dissociated PVM and Mouse Lung Particles

PVM suspension: 10 per cent perfused infected mouse lung prepared in	Treatment of suspension		Hemagglutination titer of supernate vs. mouse RBC
	NaCl added	12,000 R.P.M.	
	M	min.	
Distilled H ₂ O	0	30	128
" "	0.018	"	32
" "	0.036	"	"
" "	0.072	"	0
" "	0.145	"	"

lung particles was complete at NaCl concentrations of 0.072 M or more. With 0.036 M NaCl or less not all of the dissociated virus present in the suspension was bound by the lung particles. It is evident that the amount of dissociated PVM bound by lung particles is directly related to the electrolyte concentration.

Effect of NaCl Concentration on Recombination between Heat-Released PVM and Lung Particles.—Because the combining capacity of dissociated PVM appeared to be identical with that of heat-released virus, a study of the relationship between NaCl concentration and recombination between heat-released PVM and lung particles also was undertaken.

Mixtures of heated virus in water and lung particles in water were prepared and NaCl was added to concentrations ranging from 0.145 M to 0.018 M. The quantity of virus which combined with the lung particles was determined in the usual manner.

The results of this experiment were similar to those described in the preceding section. At NaCl concentrations of 0.072 M or more at least 50 per cent of the heated virus present combined with the lung particles, while at NaCl

concentrations of 0.036 M or less no evidence of combination between the virus and lung particles was observed.

Virus-Combining Capacity of Normal Lung Suspensions.—The experiments described in a preceding section show that as the NaCl concentration was reduced the amount of PVM bound by lung particles was also reduced. In the present experiments the virus-combining capacity of normal lung suspensions in varying concentrations of NaCl was studied.

To each of a series of twofold dilutions of a suspension of perfused normal lungs in water or in 0.15 M NaCl, 16 hemagglutinating units of PVM in 0.15 M NaCl was added. An equal quantity of PVM in 0.68 M NaCl was added to each of a series of twofold dilutions of the lung suspension in 0.68 M NaCl. Following incubation at 37°C. for 30 minutes, the combined virus was sedimented in the centrifuge and the supernates were tested for free virus by the hemagglutination technique.

TABLE V

The Effect of NaCl Concentration upon PVM-Combining Capacity of Normal Mouse Lung Suspensions

Suspension: 10 per cent perfused normal mouse lung diluted in	Virus added to each dilution	Final NaCl concentration of PVM-mouse lung particle mixture	Hemagglutination-inhibition titer of suspension
	Hemagglutinating units		
Distilled H ₂ O	16	M 0.07	8
0.15 M NaCl	"	0.15	64
0.68 " "	"	0.68	512

The results of a typical experiment are shown in Table V. It is evident that as the NaCl concentration of the normal lung suspension was increased, the hemagglutination-inhibition titer of the suspension also increased. It will be recalled that the inhibition titer of a lung suspension is taken as the highest dilution that completely combines with a constant amount of virus; this is a measure of the virus-combining capacity of the suspension (4).

As an explanation for the unexpected result obtained with high salt concentration, it seemed possible that in 0.68 M NaCl solution the lung particles were in a more dispersed state or, indeed, that some of the combining component might have become soluble. As another explanation it seemed possible that the state of the virus might be altered in hypertonic NaCl. If the virus particles became aggregated, the net effect might be to increase, falsely, the apparent virus-combining capacity of the lung suspension. However, the hemagglutination titer of a PVM suspension at NaCl concentrations ranging from 0.07 to 0.62 M did not vary and therefore it appeared that the effect of NaCl on the virus-combining capacity of lung suspensions was not to be explained on this basis. The results of these various experiments indicate

clearly that the amount of virus which is bound by a lung suspension is directly related to the NaCl concentration over the range studied.

Effect of Various Electrolytes on Combination between PVM and Lung Particles.—To determine whether the effect of NaCl upon combination between PVM and lung particles was due to either Na^+ or Cl^- ions or could be duplicated with other electrolytes, additional experiments were performed. The effect of KCl, MgCl_2 , NH_4Cl , and Na_2SO_4 , respectively, on the virus-combining capacity of lung suspensions also was studied.

To each of a series of twofold dilutions of perfused normal lung suspension prepared in one or another of the salts mentioned above, 8 hemagglutinating units of PVM in the corresponding electrolyte was added. Because of the finding that the virus-combining capacity of lung particles is greater in hypertonic solution, the electrolyte concentration in each mixture was maintained at 0.3 M in order to increase the probability of detecting small differences in the

TABLE VI

The Effect of Various Electrolytes on Combination between PVM and Mouse Lung Particles

PVM hemagglutinating units	Mixture		Hemagglutination-inhibition titer of suspension
	Suspension: 10 per cent perfused normal mouse lung	Diluent	
8	Serial dilutions	0.3 M NaCl	512
"	" "	0.3 M KCl	"
"	" "	0.3 M MgCl_2	"
"	" "	0.3 M NH_4Cl	"
"	" "	0.3 M Na_2SO_4	"
"	" "	Distilled H_2O	0

effect of various electrolytes upon the combination reaction. The virus-combining capacity of the suspension was then determined in the usual manner.

The results of these experiments, as recorded in Table VI, demonstrate that the virus-combining capacity of a lung suspension is independent of the ionic composition of the mixture. Obviously, either mono- or bivalent ions can be substituted for NaCl without influencing the capacity of lung particles to bind the virus. These results indicate that the effect of electrolytes on PVM-lung particle combination is not specific for any single ion or pair of ions, and that the effect is due to a property shared by one, at least, of each ion pair tested.

Effect of NaCl on Combination between PVM and Mouse Erythrocytes.—Sufficient evidence has been accumulated to support the concept that infection by PVM is initiated by combination between the free virus and cells of the respiratory tract (4). It appeared that a study of the influence of electrolytes upon combination of the virus with intact cells rather than with cell fragments

might provide information about some of the factors concerned in this reaction and might yield a clue concerning the mechanism. With suspensions of erythrocytes the external electrolyte environment can be readily controlled, but it would be technically difficult to control this variable with intact cells of the excised lung. Present evidence indicates that, as regards combination with PVM, mouse RBC do not differ significantly from respiratory tract cells (4). The effect of NaCl upon combination between PVM and mouse RBC was therefore studied.

A suspension of dissociated PVM in 0.25 M dextrose was added to a suspension of 50 per cent mouse RBC which had been washed in 0.25 M dextrose. The final concentration of RBC was 5 per cent. The mixture was held at room temperature; at 30 and 240 minutes aliquots were removed, the cells sedimented, and the free virus content of the supernate determined. After 240 minutes sufficient NaCl was added to raise the concentration of the mixture to 0.15

TABLE VII
The Effect of NaCl on Combination between PVM and Mouse Erythrocytes

Mixture	Treatment			Hemagglutination titer of supernate vs. mouse RBC
	NaCl added	Held at room temperature	2,000 R.P.M.	
	M	min.	min.	
PVM + RBC + 0.25 M dextrose	0	30	5	512
" " " " "	"	240	"	"
" " " " "	0.15	240 + 30	"	32
" + 0.15 M NaCl	0	0	0	512
" + RBC + 0.15 M NaCl	"	30	5	16

M. After an additional 30 minutes the virus content of the supernate was measured. A control consisted of a similar mixture to which 0.15 M NaCl was added immediately.

As shown in Table VII, combination between PVM and mouse RBC in dextrose solution did not occur even though the mixture was held for 4 hours. Yet, when NaCl was added, the virus promptly united with the erythrocytes. In other experiments the virus-combining capacity of mouse erythrocytes in hypertonic NaCl solution was compared to that in isotonic NaCl. The technique employed was similar to that described above in the section on the effect of NaCl concentration upon the combining capacity of lung particles. It was found that the combining capacity of mouse RBC in 0.73 M saline was two- to fourfold greater than that in 0.15 M NaCl.

Combination between PVM and Erythrocytes in Various Electrolyte Solutions.—Since the effect of NaCl upon virus-RBC combination appeared to be the same as upon virus-lung particle combination, the effect of various electrolytes upon the former combination was determined.

Serial twofold dilutions of a dissociated PVM suspension in water were made either in various electrolyte solutions at 0.15 M or in non-electrolyte solutions at 0.25 M. To each dilution was added an equal volume of mouse RBC suspended in a solution of corresponding composition. The hemagglutination titers were then determined.

The results are recorded in Table VIII. When hemagglutination titrations were performed in non-electrolyte solutions, agglutination of RBC did not occur. Because combination between virus and erythrocytes is the first step in the hemagglutination reaction, the absence of agglutination in non-electrolyte solutions may be attributed to failure of the virus and RBC to unite. In each of the various electrolyte solutions, on the other hand, the hemagglutination titer was identical with that obtained in the presence of NaCl. It appears that the effect of electrolytes upon combination between PVM and erythrocytes

TABLE VIII
Hemagglutination Titer of PVM in Various Electrolyte or Non-Electrolyte Solutions

Diluent	Concentration	Hemagglutination titer vs. mouse RBC	Diluent	Concentration	Hemagglutination titer vs. mouse RBC
Dextrose	0.25*	0	MgCl ₂	0.15	512
Sucrose	" "	"	Na ₂ SO ₄	"	"
NaCl	0.15	512	NaH ₂ PO ₄	"	"
KCl	"	"	Na ₂ HPO ₄	"	"
CaCl ₂	"	"	NaNO ₂	"	"

* 0.01 M phosphate added.

is a property which is shared by all of the electrolytes studied, and is not dependent upon the presence of any particular anion or cation combination. These results parallel those obtained with various electrolytes and PVM-lung particle mixtures. The similarity of the reactions suggests that the combining components in lung particles and in RBC are either the same or closely related substances. The hemagglutination titer of a PVM suspension was determined in the presence of 0.15 M NaCl in buffers of 0.1 ionic strength which ranged from pH 5 to pH 9. No significant difference in titer was observed at any of the pH levels studied. This indicates that in the presence of a sufficient concentration of electrolytes combination between the virus and RBC occurs over a wide range independently of the pH of the suspension.

The Effects of pH and Electrolyte Concentration upon Dissociation of PVM from Combination with RBC.—The release of PVM from combination with erythrocytes by heat, as previously described (1-3), destroys the combining capacity of the RBC. Release of PVM combined with erythrocytes can be achieved readily by sedimenting the RBC and causing hemolysis with water. Dissocia-

tion of combined PVM without disruption of the RBC can be effected by resuspending the virus-RBC complex in 0.25 M dextrose or sucrose buffered at pH 7.2 with 0.01 M phosphate. In general, the degree of dissociation obtained appeared to be complete.

Although the PVM-RBC complex readily dissociated in 0.25 M dextrose buffered at pH 7.2 with 0.01 M phosphate, no dissociation occurred in unbuffered 0.25 M dextrose. This observation suggested that dissociation was influenced by either the pH or the electrolyte concentration, or, possibly, by both factors

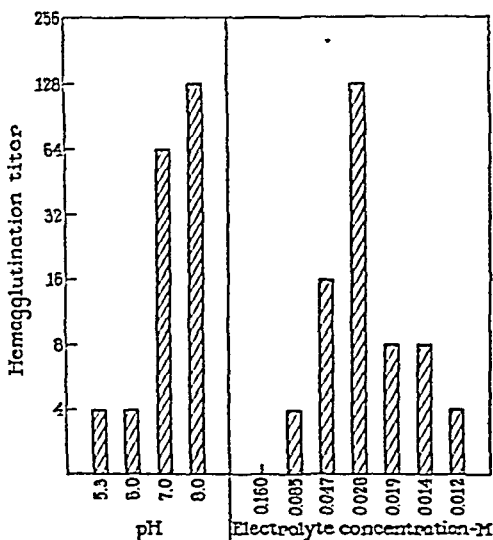


FIG. 1. The effects of varying pH and electrolyte concentration relative to each other upon dissociation of PVM from combination with mouse RBC. The pH was varied with phosphate buffers at 0.01 M. The electrolyte concentration was varied with NaCl at pH 6.0 in 0.01 M phosphate buffer.

The effect of varying pH at constant electrolyte concentration was first studied. To aliquots of a PVM preparation in saline sufficient mouse RBC were added to yield 5 per cent suspensions. After 10 minutes the mixtures were centrifuged, and the RBC washed twice in unbuffered 0.25 M dextrose. The sedimented cells were resuspended to volume in 0.25 M dextrose solutions buffered with 0.01 M phosphate at pH 5.3, 6.0, 7.0, and 8.0, respectively. After 30 minutes the cells were again sedimented, and the amount of virus in the supernates was measured by the hemagglutination technique.

The results of a typical experiment are shown in Fig. 1. It will be seen that a significant amount of the virus dissociated from the erythrocytes only when the cells were resuspended in dextrose buffered at pH 7 or 8. Therefore, it appears that at constant electrolyte concentration dissociation is a function of the pH of the suspension.

In order to investigate the effect of varying electrolyte concentration at constant pH upon dissociation, a modification of the procedure described above was employed.

Virus-RBC complexes were formed in saline. The erythrocytes then were washed twice in unbuffered dextrose and resuspended to volume in 0.25 M dextrose buffered at pH 6.0 with 0.01 M phosphate. NaCl was added to the suspension in concentrations ranging from 0.15 M to 0.01 M. After 30 minutes the cells were again sedimented, and the amount of virus present in the supernate was measured in the usual manner.

The results of a typical experiment also are shown in Fig. 1. It is evident that at pH 6.0 dissociation was maximal at 0.028 M electrolyte concentration and that either lower or higher concentrations markedly reduced dissociation. These results indicate that dissociation of PVM from combination with RBC is a function of both the electrolyte concentration and the pH of the suspension. A study of the behavior of PVM-RBC stromata complexes with respect to the effect of electrolyte concentration showed that combination and dissociation also could be caused to occur by appropriate regulation of this variable.

Sequential Cycles of Combination and Dissociation of PVM and Mouse Erythrocytes.—It is well established that mouse erythrocytes contain a substance with which PVM combines and that spontaneous dissociation of the virus from combination does not occur (1-5). Nonetheless, dissociation and recombination can be achieved by varying the electrolyte concentration. It appeared probable that additional information about the PVM-RBC reaction could be obtained by determining whether or not either the virus or the red blood cell was altered as a result of combination and dissociation.

To a suspension of dissociated PVM in 0.25 M sucrose was added sufficient NaCl to yield a 0.15 M solution. Packed mouse erythrocytes, washed in saline, were then added to a concentration of 5 per cent. Preliminary experiments indicated that this amount of RBC would combine with all of the virus present in the preparation used. The mixture was held 15 minutes, the cells sedimented, the supernate removed, and its hemagglutination titer determined. The packed RBC were resuspended to volume in 0.25 M sucrose, buffered at pH 7.2 with 0.01 M phosphate. After 15 minutes the RBC were again sedimented, an aliquot of the supernate removed, and its hemagglutination titer measured. To cause another cycle of combination and dissociation between virus and RBC, the mixture was shaken, sufficient NaCl was added to restore the concentration to 0.15 M, and the cells were again sedimented after 15 minutes. The supernate was removed and titered as before. Additional cycles were performed by a repetition of these steps.

The results of a typical experiment in which 3 cycles of combination and dissociation were carried out are shown in Table IX. They demonstrate clearly that in the presence of sucrose and NaCl combination between PVM and RBC occurs, while in the presence of sucrose alone dissociation of the complex results. This cycle can be repeated at will by appropriate regulation of the NaCl concentration and the pH of the mixture. These results indicate

that PVM, unlike the influenza group of viruses (6), does not inactivate or destroy the "receptor" of erythrocytes, and repeated cycles of combination and dissociation can occur without causing demonstrable alteration of the combining capacity of either the virus or the RBC.

The Effect of Low Electrolyte Concentration upon Hemagglutination with Influenza Viruses.—It was reported recently (14) that the concentration of NaCl markedly affects the hemagglutination reaction between the PR8 strain and human erythrocytes. In the present study preliminary observations demonstrated that, whereas hemagglutination with PVM and mouse RBC could be completely inhibited at electrolyte concentrations of 0.01 M or less, to achieve the same result with influenza viruses and chicken RBC it was necessary to reduce the electrolyte concentration to 0.001 M. This finding raised two

TABLE IX

Sequential Cycles of Combination and Dissociation between PVM and Mouse Erythrocytes

Step No.	PVM suspension in 0.25 M sucrose	Treatment of sedimented RBC			Hemagglutination titer of supernate vs. mouse RBC
		Resuspended in sucrose*	NaCl added	2,000 r.p.m.	
1	Supernate	M	M	min.	
2	" + RBC	0	0	0	256
3	Resuspended sediment from No. 2	"	0.15	5	0
4	" " " "	0.25	0	"	256
5	" " " "	"	0.15	"	0
6	" " " "	"	0	"	256
7	" " " "	"	0.15	"	0
	" " " "	"	0	"	256

* Buffered at pH 7.2 with 0.01 M phosphate.

technical difficulties. Firstly, certain lots of chicken RBC are unstable and spontaneously agglutinate in 0.25 M dextrose containing 0.001 M phosphate. However, it was found that by holding the RBC in dextrose solution overnight at 4°C. this undesirable effect could be eliminated. Secondly, the pH of 10 per cent suspensions of chicken RBC in 0.001 M phosphate-buffered dextrose is only slightly above 6, whereas the pH of such cells in dextrose buffered with 0.01 M phosphate is 7.2. Therefore, in order to interpret the experiments to be carried out, it was necessary first to study the effect of pH upon the hemagglutination reaction with influenza viruses and chicken RBC. It was readily demonstrated that in saline, buffered at pH 6, 7, and 8, respectively, with phosphate buffer (0.1 ionic strength) the hemagglutination titer and the rate of elution of virus were not appreciably affected. In order to determine the effect of electrolyte concentration upon the hemagglutination titer of influenza virus, the following experiment was performed.

Pools of infected allantoic fluid were dialyzed at 4°C. against large volumes of water buffered at pH 7.2 with 0.001 M phosphate. Serial twofold dilutions were made in 0.25 M dextrose similarly buffered. To each dilution was added an equal volume of a 1 per cent suspension of chicken RBC in 0.25 M dextrose containing 0.001 M phosphate. Simultaneously, aliquots of the dialyzed virus preparations were titrated in saline in the usual manner.

The results of typical experiments are recorded in Table X. It will be noted that in each instance hemagglutination was completely suppressed in solutions of low electrolyte concentration. That this result was not due to an irreversible effect of the dextrose solution upon either the virus or the RBC was readily established. When the NaCl concentration of the virus and RBC suspension in dextrose was raised to 0.15 M, the hemagglutination titer of the virus was

TABLE X

The Effect of Various Electrolytes upon the Hemagglutination Titer of Influenza Viruses

Virus dilutions and RBC prepared in solutions of*	Concentration	Hemagglutination titer vs. chicken RBC			
		PR8	FM1	Lee	B1103
Dextrose	0.25	0	0	0	0
Sucrose	"	"	"	"	"
NaCl	0.15	4096	512	2048	1024
KCl	"	2048	"	"	"
MgCl ₂	"	1024	128	1024	2048
LiNO ₃	"	"	512	"	"
Na ₂ HPO ₄	"	512	256	4096	"
NaH ₂ PO ₄	"	1024	4	4	0
Na ₂ SO ₄	"	"	256	2048	2048

* Each solution contained 0.001 M phosphate.

undiminished, and both adsorption and elution of the virus occurred in the usual manner. These experiments demonstrate that hemagglutination with influenza viruses, like that with PVM, can be completely inhibited if the electrolyte concentration is sufficiently low.

The Effect of Various Electrolytes upon Hemagglutination with Influenza Viruses.—Because hemagglutination with influenza viruses was completely inhibited if the electrolyte concentration was held at 0.001 M but was demonstrable in unaltered degree upon the addition of 0.15 M NaCl, it was of interest to determine the effect of various other electrolytes upon the reaction.

Pools of infected allantoic fluid were dialyzed against water buffered at pH 7.2 with 0.001 M phosphate. Serial twofold dilutions were made in a solution of the desired electrolyte at 0.15 M and 0.001 M phosphate. To each dilution was added an equal volume of chicken RBC suspended in a solution of corresponding composition. The hemagglutination titers were then determined. Spontaneous agglutination of RBC in the solutions used was not observed.

The results of these experiments, as shown in Table X, demonstrate that, as in the case of PVM, hemagglutination with influenza viruses is not dependent upon the presence of any single ion or ion pair investigated but is a function of the electrolyte concentration. No detailed investigation of the variability shown in the hemagglutination titer in various electrolytes was undertaken. The unusually low titer of the Lee, FM1, and B1103 strains in 0.15 M NaH_2PO_4 is attributable to the fact that the hemagglutination titer of each of these strains was greatly diminished at pH 5.0, and the pH of the NaH_2PO_4 solution employed was 4.7.

The Effect of Low Electrolyte Concentration upon Combination between Influenza Viruses and RBC.—It was important to determine whether the results described above were due to a failure of the virus to combine with erythrocytes, or to a failure of erythrocytes which had combined with virus to agglutinate.

Infected allantoic fluids were dialyzed at 4°C. against distilled water buffered at pH 7.2 with 0.001 M phosphate. Dextrose was added to a concentration of 0.25 M. Packed RBC, washed in 0.25 M dextrose containing 0.001 M phosphate, were added to yield a 5 per cent suspension. The mixtures were held at room temperature and at intervals aliquots were removed, centrifuged, and the hemagglutination titer of the supernate measured. As a control, 0.15 M NaCl and sufficient RBC to yield a 5 per cent suspension were added to an aliquot of dialyzed virus.

The results of a typical experiment are recorded in Table XI. At 10 minutes all of the virus present in the control mixture had combined with the erythrocytes. In contrast, in dextrose solution, there was no significant reduction in the hemagglutination titer of aliquots removed at the indicated intervals. Similar experiments with dialyzed allantoic fluid infected with the PR8 strain gave identical results. Because the virus concentration was measured in twofold dilution series, as much as 50 per cent of the virus could have combined with the RBC without causing more than a one tube reduction in titer. In addition, it should be emphasized that the two reactions, *i.e.* combination and elution, occur simultaneously and at different rates.

In order to estimate more precisely the amount of virus which might have combined with chicken erythrocytes in dextrose, the following experiment was performed.

To a Lee virus preparation which had been dialyzed, as described above, was added dextrose to 0.25 M. Packed RBC, washed in buffered 0.25 M dextrose, were added to yield a 5 per cent suspension. The mixture was held at room temperature and at intervals of 10, 30, 60, and 120 minutes aliquots were removed and the RBC sedimented. The sediments were washed twice in dextrose and resuspended to volume in saline. The resuspended RBC were held at room temperature for 2 hours to allow dissociation of the virus to occur. The RBC were again sedimented and the hemagglutination titers of the supernates determined.

It was found that approximately 10 per cent of the virus originally present in the dextrose suspension was recovered from the RBC by spontaneous dis-

sociation in saline at each of the time intervals tested. Therefore, it appears evident that at low electrolyte concentrations influenza virus combines with erythrocytes in greatly diminished amount.

The effect of electrolyte concentration upon combination between RBC and influenza virus which had been treated so as to eliminate the phenomenon of elution (15) also was investigated.

Allantoic fluid, infected with the Lee strain, was heated at 56°C. for 30 minutes. The preparation then was dialyzed as in previous experiments. Dextrose was added to 0.25 M.

TABLE XI

The Effect of Non-Electrolyte Solutions on Combination between Influenza Virus and Chicken RBC

Mixture			Treatment of mixture		Hemagglutination titer of supernate vs. chicken RBC
Virus dialyzed vs. phosphate 0.001 M	Chicken RBC	Diluent	Held at room temperature for	2,000 R.P.M.	
	<i>per cent</i>		<i>min.</i>	<i>min.</i>	
Lee	0	NaCl 0.15 M	0	0	512
"	5	" " "	10	5	0
"	"	Dextrose 0.25 M, phosphate 0.001 M	"	"	512
"	"	" "	30	"	256
"	"	" "	60	"	512
"	"	" "	120	"	"
Lee heated*	0	" "	0	0	256
" "	5†	" "	10	5	128
" "	"	" "	30	"	"
" "	"	" "	60	"	"
" "	"	NaCl added to 0.15 M	60 + 10	"	0

* 56°C. for 30 minutes after dialysis.

† Dialyzed vs. 0.25 M dextrose containing 0.001 M phosphate.

In order to maintain the pH as close to physiological limits as possible, chicken RBC were dialyzed at 4°C. against large volumes of 0.25 M dextrose buffered at pH 7.2 with 0.001 M phosphate. The pH of the resultant suspension was 6.8. The dialyzed RBC were then added to the virus preparation to yield a 5 per cent suspension. The mixture was held at room temperature and at intervals aliquots were removed, centrifuged, and the hemagglutination titer of the supernate measured. After 60 minutes NaCl was added to 0.15 M and the concentration of uncombined virus in the supernate measured after an additional 10 minutes.

The results of a typical experiment are shown in Table XI. It will be seen that influenza virus treated so as to eliminate elution also combined in greatly diminished amounts with chicken RBC at low electrolyte concentration. However, prompt combination resulted when 0.15 M NaCl was added to the mixture.

Influenza Virus-Erythrocyte Dissociation in Non-Electrolyte Solutions.—The effect of isotonic non-electrolyte solutions upon spontaneous dissociation of influenza virus from erythrocytes was determined. In two recent papers (14, 16) certain effects of various electrolytes and of electrolyte concentration upon the elution of influenza viruses from human RBC were reported.

To pools of allantoic fluid infected with the Lee strain were added RBC in buffered saline to yield a 5 per cent suspension. After 5 minutes the suspensions were centrifuged and the cells washed in unbuffered 0.25 M dextrose. One aliquot was resuspended to volume in a solution containing 0.15 M NaCl, 0.25 M dextrose, and 0.001 M phosphate. The other was resus-

TABLE XII

The Effect of Low Electrolyte Concentration upon Dissociation of Influenza Virus from Chicken RBC

Mixture		Treatment of sediment			Hemagglutination titer of supernate vs. chicken RBC
Virus strain	Chicken RBC	Resuspended in	Held at room temperature for	2,000 r.p.m.	
	<i>per cent</i>	<i>M</i>	<i>min.</i>	<i>min.</i>	
Lee	5	0.15 NaCl 0.25 dextrose 0.001 phosphate	15	5	64
"	"	" "	45	"	128
"	"	" "	90	"	256
"	"	" "	120	"	"
"	"	0.25 dextrose 0.001 phosphate	15	"	16
"	"	" "	45	"	32
"	"	" "	90	"	64
"	"	" "	120	"	"
"	0	0	0	0	512

pended to volume in 0.25 M dextrose containing 0.001 M phosphate. At intervals samples were withdrawn and after centrifugation the hemagglutination titer of the supernates was determined.

The results of typical experiments are presented in Table XII. It was found that, unlike PVM, influenza virus did not rapidly dissociate from erythrocytes upon reduction of the electrolyte concentration of the suspension. On the contrary the rate of spontaneous dissociation was considerably retarded at low salt concentrations. The results of similar experiments with the B1103 strain were identical. It is of interest that high salt concentrations have been found to increase the elution rate (17). A comparable reduction in the rate of elution of the Lee strain from human RBC in low electrolyte concentration

was also demonstrated. In order to determine whether or not the different effects of low electrolyte concentrations upon PVM- and influenza virus-RBC combinations were due to a difference in the properties of the virus strains or species of erythrocytes employed in these experiments, attempts were made to induce rapid dissociation at low electrolyte concentrations with PR8-chicken and human RBC combinations. In addition, the effect of low electrolyte concentrations upon combinations between human, chicken, and mouse RBC with Lee virus, heated at 56°C. for 30 minutes, was investigated. In each instance rapid dissociation comparable to that obtained with PVM-mouse RBC combinations, was not demonstrated. Moreover, with heated Lee virus low electrolyte concentrations caused no dissociation even though the mixture was held for 2 hours at room temperature. These findings support the concept that the marked differences in the reactions of PVM and influenza

TABLE XIII

The Relationship between Influenza Virus Concentration, Electrolyte Concentration, and Hemagglutination

Hemagglutinating units added	Electrolyte concentration, <i>M</i>									
	0.146	0.073	0.037	0.019	0.010	0.005	0.003	0.002	0.0015	0.0012
8	4*	3	3	1	±	0	0	0	0	0
32	4	3	3	3	3	3	2	0	0	0
128	4	4	3	3	3	3	3	3	2	0
0	0	0	0	0	0	0	0	0	0	0

* Degree of hemagglutination.

viruses with erythrocytes at low electrolyte concentrations are attributable to fundamental differences in the viruses themselves.

Relationship between Concentrations of Influenza Viruses and Electrolytes and Combination with RBC.—In order to assess the effects of varying the concentration of both influenza viruses and electrolytes relative to each other upon combination with RBC, the following experiment was performed.

Replicate serial twofold dilutions of a 1.2 *M* solution of NaCl were made and to each dilution series was added either 8, 32, or 128 hemagglutinating units of dialyzed Lee virus. Chicken RBC were then added to give 0.5 per cent suspensions. The RBC and virus suspensions, as well as the NaCl dilutions, were prepared in 0.25 *M* dextrose containing 0.001 *M* phosphate. After 1 hour the degree of hemagglutination in each mixture was recorded.

The results of a typical experiment are shown in Table XIII. It will be noted that, as indicated by the absence of hemagglutination, the electrolyte concentration at which influenza virus-RBC combination was inhibited was inversely related to the amount of virus present in the mixture. Similar

experiments were carried out with the PR8 and B1103 strains and in both instances an identical relationship was found. In experiments of this type with PVM the effect of virus concentration was much less striking. Whereas with influenza viruses a fourfold increase in the amount of virus allowed approximately a fourfold decrease in the electrolyte concentration, with PVM a 32-fold increase in the amount of virus permitted only about a twofold decrease in electrolyte concentration. It seems probable that the inverse relationship between virus and electrolyte concentration can be attributed to the effects of these variables upon the combination equilibrium with erythrocytes. However, it is possible that it reflects an inhomogeneity in the virus population itself. Recent evidence (17) suggests that such an inhomogeneity in one property of influenza virus particles may exist.

DISCUSSION

Combination between viruses and host cell components is a well known phenomenon (18) which has been studied by numerous investigators. It is well established that such combinations occur with bacterial (19), plant (20), and animal viruses (3, 21). One example of combination between certain animal viruses and host cells is provided by the hemagglutination reaction which during recent years has received intensive study (1, 6, 22-26).

That variation of electrolyte concentration causes striking effects upon the reactions between pneumotropic viruses and cell components appears evident from the results of this study. In earlier studies (14, 16) the effects of electrolytes on reactions between influenza viruses and human erythrocytes were reported. Under conditions of constant osmotic pressure, not only is hemagglutination with either PVM or the influenza viruses completely inhibited at low electrolyte concentration, but also adsorption of the viruses on erythrocytes is greatly diminished. Of more interest is the fact that dissociation of PVM from combination with either lung particles or erythrocytes is brought about at low electrolyte concentration (*i.e.*, 0.01 M). This occurs with infectious virus and also with virus which has been heated at 70°C. It is apparent, therefore, that in the case of PVM dissociation is not dependent upon the integrity of heat-labile components of the virus. In contrast, dissociation of influenza virus from erythrocytes is adversely affected even by very low concentrations of electrolytes (*i.e.*, 0.001 M); with infectious virus the rate of elution is definitely decreased; with virus heated at 56°C. no dissociation occurs. Because of this it seems probable that the forces which hold PVM in combination with cell components are different from those responsible for combination between influenza virus and cell components and it is evident that they are oppositely influenced by alterations in electrolyte concentration.

It should be emphasized that heretofore it has not been possible to cause dissociation of either PVM-lung particle or PVM-erythrocyte combinations

by any procedure other than those which both inactivate the virus and irreversibly alter the combining component (5). As a consequence, it was not possible previously to determine whether PVM shares with influenza and certain other viruses the peculiar capacity to alter the components with which it combines. That PVM does not alter the combining component of erythrocytes seems apparent from the results obtained. Free infectious virus can be caused to combine with and dissociate from erythrocytes repeatedly without measurably affecting their combining capacity. Thus, it appears that the enzyme-like activity of influenza viruses (6) finds no counterpart in the activities of PVM.

In this connection it seems pertinent to point out that in nature PVM is a latent agent which, it appears, does not induce manifest disease unless the equilibrium between virus and host is upset by experimental manipulation (27). It may be that the capacity of the agent to remain latent and to induce only inapparent infection under normal environmental conditions is related to the fact that the virus is incapable of dissociating spontaneously from combination with cell components. It seems evident that an agent which could not readily become separated from an intact cell might find difficulty in migrating to and initiating infection of another cell. The fact that uncombined PVM can be obtained from the intact lungs of experimentally infected animals (4, 5) is not evidence against this hypothesis. Not only does manifest infection with the virus lead to necrosis of lung tissue cells which undoubtedly results in the release of intracellular enzymes, but also the combining component of such cells is more readily destroyed by proteolytic enzymes than is the virus (4). Under these circumstances it would be expected that some free virus could be demonstrated in heavily infected intact lungs.

Both in the case of PVM (5) and the influenza viruses (28, 29) it appears that hemagglutination titrations provide a measure of the concentration of free or uncombined virus but give no direct indication of the amount of combined virus which may be present. In both instances also the hemagglutination reaction may be markedly affected by the composition of the medium in which it occurs. As regards the effect of low electrolyte concentrations, the combining reactions of PVM are approximately 10 times more sensitive than are those of the influenza viruses.

It is obvious that the low electrolyte concentrations which are required to cause inhibition of the reactions between the viruses and cell components under study are far outside of the physiological range and undoubtedly would not be encountered in living tissues. Nevertheless, by exceeding physiological limits, it has been possible to learn more of the factors which are operative in the physiological range. The results obtained suggest that the attractive forces which cause either PVM or influenza viruses to combine with particular cell components are highly active at physiological concentrations of electrolytes.

Moreover, they suggest that in the case of PVM such forces appear to be ionic in nature inasmuch as it is evident that they depend upon the ionic concentration and the pH. The findings are consistent with the supposition that the PVM-cell component complex is of the nature of a weak salt.

SUMMARY

Combination between PVM and erythrocytes as well as between the influenza viruses and erythrocytes is inhibited at low electrolyte concentrations. Combination between PVM and lung particles as well as between the virus and erythrocytes can be dissociated in solutions of low electrolyte concentration. The rate of elution of influenza virus is decreased under similar conditions. PVM can combine with and be dissociated from erythrocytes repeatedly without affecting the combining capacity of the cells and does not possess an enzyme-like activity similar to that of the influenza viruses. Because dissociation depends on electrolyte concentration and pH, it appears that the PVM-cell component complex may be in the nature of a weak salt.

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THE SUSCEPTIBILITY OF THE HAMSTER TO MOUSE ENCEPHALOMYELITIS VIRUS

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Only the mouse has been shown to be susceptible to the original (1) strains of mouse encephalomyelitis. The FA and GDVII (2) strains have a wider host range although they, too, have been found to occur naturally only in the mouse. When they have been detected in other species the circumstances suggested infection from mice (3). In our experience virus has never been recovered from normal hamsters nor has hamster serum reacted with Theiler viruses. Indeed it is frequently used in this laboratory as a negative control. On the other hand, in experiments reported some years ago (4) indirect evidence was secured of infection of hamsters without manifest signs and this has now been investigated. It has been learned that unmodified strains do cause inapparent infections and that strains experimentally manipulated may become fully pathogenic, capable of inducing both paralysis and encephalitis.

EXPERIMENTAL

Methods.—The virus used in the present experiments, strain 4727, was isolated from the intestinal contents of a mouse from the laboratory-bred colony in 1945 and has been extensively used in mouse experiments. There has been no change in the signs of infection it induces, which are those Theiler originally described (1), although the mouse infectivity titer has increased. The estimated median effective dose of mouse brain is now approximately 10^{-5} . A stock 5 per cent mouse brain suspension of virus in 0.85 per cent salt solution containing 10 per cent beef infusion broth has been employed in neutralization tests, the suspensions being customarily stored at -70°C . until used. All the suspensions have been cultured aerobically by streaking blood-agar plates. The neutralization tests have been performed by mixing equal parts of twofold dilutions of the virus and serum and incubating the mixtures for 3 hours at room temperature. The inoculations have been intracerebral. The use of twofold dilutions and the moving averages method (5) for estimating the median effective dose have been used to analyze the results of tests.

The hamsters (*Mesocricetus aureatus*) were from the laboratory colony. The mice were albinos of the Albany standard strain. The hamsters were observed for 30 days, the mice for 35 days except for neutralization and titration tests for which 28 and 21 day periods have been adopted.

Survival of Virus in Suckling Hamsters.—The initial experiments were made to determine whether virus survives in the brains of immature hamsters inoculated intracerebrally (Table I). Two litters were used, one 4 and the other 9 days old. Each animal was inoculated with a 20 per cent suspension of mouse

brain virus. Thereafter, at intervals of 2 or more days, a single hamster was killed and its brain removed and tested for the presence of virus by the inoculation of 10 to 12 gm. mice. The first litter was sampled on five occasions between the 3rd and 14th days. Virus was present up to and including the 10th day. The second litter was sampled from the 3rd to the 30th days and virus found as late as the 14th day. None of the hamsters exhibited any signs of illness.

Serial Passage in Hamsters.—The next experiment was planned to determine whether the pathogenicity of the virus could be increased by serial, blind passages in suckling hamsters (6). Animals were inoculated as before, sacrificing each generation, one or more litters, on the 4th or 5th day. Parts of the brain and cord were preserved for histologic examination; the remainder

TABLE I

Survival of Virus and Occurrence of Lesions in Suckling Hamsters Inoculated with Mouse Encephalomyelitis Virus

Days	Response of test mice		Lesions of CNS of hamsters
	Litter 1	Litter 2	
3	9/10	10/10	None
5	9/9	11/11	Acute encephalomyelitis
7	10/10	10/10	None
10	8/10	1/8	Scanty meningitis
14	0/10	2/8	Scanty meningitis
22		0/9	None
30		0/10	Scanty meningitis

The denominator indicates the number of mice inoculated with hamster brain suspensions, the numerator, the number paralyzed or dead.

was suspended in four parts of broth-saline and inoculated into the next generation of hamsters. Each inoculum was also tested in groups of young mice. Nine passages were made. In none of the 103 hamsters was paralysis seen. Virus was present throughout five generations. All the test mice inoculated with hamster brain of the first four generations were paralyzed. Brains of the last four generations induced paralysis in but one of the forty test mice. The progressive loss of infectivity was paralleled by the disappearance of lesions which were present in the second and third generations but not after the fifth passage (Table II).

These results confirmed the previous experiment and supplemented it by showing that strain 4727 was capable of causing lesions in suckling hamsters. The method failed to adapt the virus to hamsters possibly because infectivity titer was not maintained.

Alternate Hamster-Mouse Passage.—Since preliminary experiments had

indicated that infectivity of the hamster passage virus for mice could be maintained by alternating the species we next undertook to alternate mouse and hamster passage, testing for the presence of virus and lesions as before.

The hamsters were litters between 4 and 11 days of age and the mice, usually ten in number, were 10 to 12 gm. albinos of the Albany standard strain. The inoculum was uniformly 0.03 ml. of a 20 per cent brain suspension. Some of the suspensions were prepared and injected immediately upon harvesting the brains and others after storage in 50 per cent glycerol. The hamsters were sacrificed 5 to 7 days following inoculation during the first three generations and when paralyzed in the subsequent generations. The mouse brains were harvested from the first mice of each group to succumb.

TABLE II
Blind Passage of Encephalomyelitis Virus in Immature Hamsters

Passage	Response of hamsters	Mouse infectivity of hamster brains	Lesions in hamsters	
			CNS	Spinal muscles
1	0/15	9/9	Acute encephalomyelitis	+
2	0/15	11/11	Acute encephalomyelitis	+
3	0/6	—	None	+
4	0/14	9/9	Scanty meningitis	
5	0/12	3/9	None	+
6	0/17	0/10	None	
7	0/10	1/10	None	
8	0/14	0/10	None	
9	—	0/10	None	

The denominator indicates the number of hamsters or mice inoculated, the numerator, the number paralyzed or dead.

All the mice became paralyzed. The uniformly high infectivity of the hamster brains was verified by titrations in mice (Table III). No signs of infection were observed in the first two hamster generations but the third passage seemed to introduce a change. One of the two litters that had been inoculated grew poorly and a number of the animals died. The brain chosen for passage proved to have a slightly lower mouse titer but six of eight immature hamsters inoculated with it nevertheless became paralyzed. This was the first appearance of paralysis. It occurred regularly in each of the six subsequent generations. In the fifth and sixth hamster passages, all the animals exhibited symptoms of encephalitis as well. This appeared on the 5th post-injection day and was followed in a third of the animals by paralysis. The non-paralyzed survivors were stunted.

Thus there were two obvious reasons for suspecting that the virus had been modified, the development of paralysis and subsequently and independently the appearance of encephalitis. Further evidence of alteration was secured

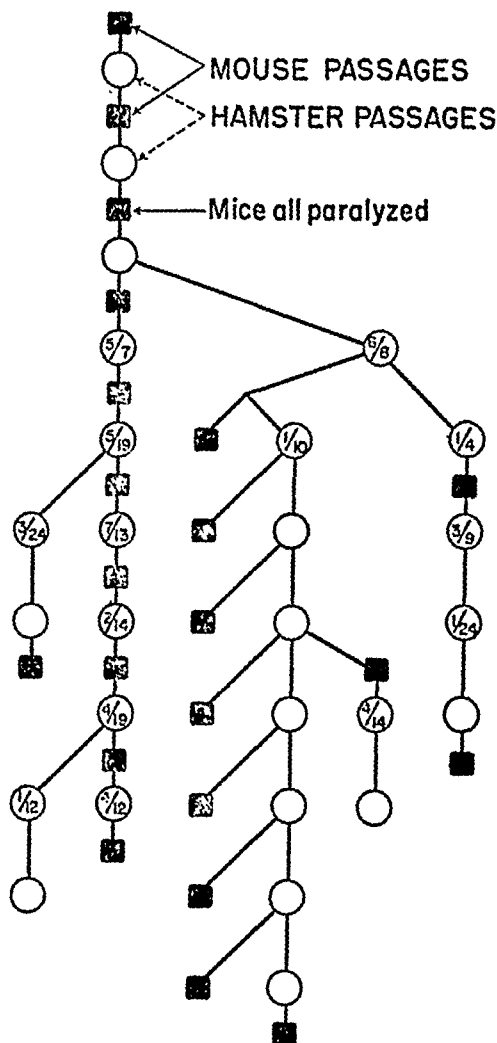


FIG. 1. Modification of mouse encephalomyelitis virus by alternating mouse-hamster passages. The numerators show the number of test hamsters that were paralyzed. The empty circles indicate silent infection. Note the rapid loss of pathogenicity following serial hamster transfers and its restoration by a single mouse passage. Note also the persistence of mouse infectivity throughout nine serial hamster passages.

by serially transferring the virus in hamsters. Whereas the original strain died out after four or five hamster transfers the modified virus persisted indefinitely. Fig. 1 illustrates some of the tests, including the successful transmission of the agent through nine hamster transfers. It may be noted that six of these were without signs of disease but that paralysis was again induced

by a single mouse passage. The restoration of pathogenicity by a single mouse passage occurred whenever tried, on all of four different occasions.

Table III lists the results of tests for infectivity titer of mouse and hamster brain suspensions from various stages of the experiment. Infectivity for mice remained high throughout five serial hamster passages. Brains of the test mice of the fifth hamster generation induced paralysis in four of fourteen young hamsters. The sixth serial passage in hamsters, completely without

TABLE III

Mouse-Infectivity Titer of Strain 4727 before and after Mouse Passage, Alternating Mouse-Hamster, and Serial Hamster Passages

	Hamster brain dilutions tested											$m(10^5)^*$
	1/1000	1/2000	1/4000	1/8000	1/16,000	1/32,000	1/64,000	1/128,000	1/256,000	1/512,000	1/1,024,000	
At beginning of experiment.....	12/12	10/12	9/12	12/12	6/12	9/12	4/12	—	—	—	—	2.9
After 5 mouse passages.....	8/8	8/8	8/8	7/8	7/8	8/8	8/8	8/8	3/8	2/8	—	0.4
After 9 mouse passages.....	8/8	8/8	8/8	7/8	7/8	3/8	6/8	4/8	1/8	0/8	2/8	1.1
After 4 hamster mouse passages..	6/6	6/6	6/6	5/6	4/6	6/6	6/6	4/6	2/6	—	—	0.5
After 9 hamster mouse passages..	6/8	6/8	7/8	4/8	0/8	2/8	1/8	1/8	3/8	0/8	—	14.04
After 5 hamster passages of the hamster-adapted strain.....	8/8	5/8	3/8	4/8	1/8	1/8	0/8	0/8	—	—	—	25.0

The denominator indicates the number of test mice inoculated with the particular dilution, the numerator, the number paralyzed or dead.

* Median effective dose (5).

signs of disease, nevertheless induced a sharp antibody response (Table IV), the serum activity equalling that seen in mice hyperimmunized with encephalomyelitis virus.

It was thus evident that alternate mouse-hamster passages had modified the virus of mouse encephalomyelitis. The modified virus differed from the parent virus in its pathogenicity for the hamster, a greater ability to maintain itself throughout serial hamster transfers, and a kind of residual pathogenicity which could be activated by a single mouse passage.

Signs of Encephalitis in Hamsters.—In our experience with the OT strain of mouse encephalomyelitis, encephalitic signs have been rarely seen. With

strain 4727, mice develop only flaccid paralysis, usually of the hind legs. The shortest incubation period is 6, rarely 5, days with additional animals becoming paralyzed in subsequent weeks. The longest incubation period we have observed was 44 days.

A comparison was made of the incubation periods in 114 paralyzed mice and 38 paralyzed hamsters inoculated with the suspensions used in the alternating hamster-mouse passages (Fig. 2). The incubation period of the mice varied

TABLE IV

Development of Serum Antibodies in Hamsters Inoculated with Hamster-Adapted Mouse Encephalomyelitis Virus without Obvious Response

Serum	Response of test mice to indicated virus dilutions plus serum or broth salt										m(10 ³)	
	1/250	1/500	1/1000	1/2000	1/4000	1/8000	1/16,000	1/32,000	1/64,000	1/128,000		1/256,000
Sera of inoculated hamsters (30 days)*.....			3/8	5/8	3/8	2/8	1/8	1/8	—			100
Sera of normal hamsters....			8/8	8/8	7/8	4/8	6/8	3/8	—			5.3
Broth salt.....			8/8	7/8	6/8	6/8	5/8	2/8	1/8			5.5
Sera of inoculated hamsters (30 days)†.....	8/8	7/8	4/8	2/8	3/8	0/8	0/8	0/8	0/8	0/8	—	84.14
Convalescent hamster sera (30 days)§.....	7/8	3/8	2/7	1/8	4/8	2/8	0/8	0/8	0/8	0/8	—	193.2
Normal hamster sera.....	—	8/8	7/7	6/8	6/8	3/8	2/7	0/8	0/8	1/8	—	14.26
Broth salt.....	—	—	8/8	4/8	4/8	5/8	3/8	2/8	1/8	0/8	1/8	12.50

The denominator indicates the number of hamsters or mice inoculated, the numerator the number paralyzed or dead.

* The sixth serial hamster passage of modified virus.

† The third serial hamster passage of modified virus.

§ The sixth alternate mouse-hamster passage of modified virus.

from 5 to 18 days with a mean of 9.1 days. The hamsters were paralyzed between the 5th and 9th days with a mean of 6.1 days. Of the thirty-eight paralyzed hamsters, twenty-eight were paralyzed in one or both hind legs and six in the fore legs, roughly the same as occurs in mice.

The lesions in mice are essentially the same as those described by Olitsky and Schlesinger (7) but the lesions in hamsters differ greatly in degree. The reaction in the brain and brain stem resembles that seen in mice but the punctate, destructive lesions of the anterior horns of the spinal cords of mice have rarely been duplicated in hamsters despite the frank, flaccid paralysis which has been so common. On the other hand, changes have been regularly found

in the striated muscles about the vertebral column. The muscle lesions consist of hyaline degeneration shortly followed by intensive regeneration of young muscle cells and the appearance of endothelial phagocytes. Such lesions have been found in all the hamsters in which virus was demonstrated whether paralyzed or not, as well as in paralyzed mice. Similar lesions have been noted in hamsters inoculated with MM virus and in mice infected with Theiler viruses by Pappenheimer (8) whose preparations we have examined.

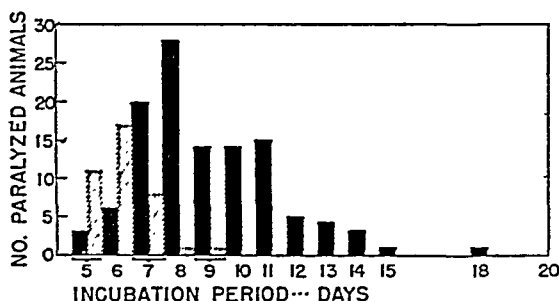


FIG. 2. Incubation periods of paralysis in mice (solid columns) and hamsters (shaded columns) inoculated with hamster-adapted mouse encephalomyelitis virus.

TABLE V

Neutralization of Hamster-Adapted Virus by Normal and Immunized Mouse Sera

Serum	Response of test mice							T ₅₀ (10%)
	1/1000	1/2000	1/4000	1/8000	1/16,000	1/32,000	1/64,000	
Normal mouse								
10/7/47.....	6/8	3/7	1/8	0/8	0/8	—	—	59.98
GDVII mouse								
3/11/47.....	5/8	2/8	2/8	0/8	0/8	—	—	75.86
FA mouse								
5/27/47.....	4/8	6/8	0/8	1/8	0/8	—	—	79.43
Broth salt.....	—	—	6/8	7/8	7/8	2/8	2/7	3.5

They are similar to those recently reported in mice inoculated with a newly isolated and unidentified agent (9). Histologic studies of the muscle lesions and the related nerves are underway.

Identification of the Virus.—The strain of virus used in these experiments is regularly neutralized by adult mouse serum, the titer of which increases with the age of the animals. The hamster-passaged strain induces similar antibodies (Table V). Moreover, the behavior and anatomical response of mice inoculated with either the parent or hamster-passaged strain is unchanged. These observations lead us to believe that the virus in hamsters and mice is the same.

DISCUSSION .

Baker has recently called attention to the ease with which certain viruses may be adapted to resistant hosts by alternately passing them through a susceptible species (10), adapting rinderpest and hog cholera to the rabbit in this way. Koprowski, James, and Cox have also used the method and refer to earlier studies (11). The present results are a further successful application of this principle. The principle is doubtless useful in the manipulation of viruses and one is tempted to speculate on its occurrence and epidemiologic importance in nature.

A particularly revealing observation was made by Coffey while studying the characteristics of vaccinia during repeated transfers through chicken embryo medium (12). Coffey noted that the titer of her strain of vaccinia diminished rapidly during serial tissue culture transfers but could be promptly restored by two rabbit passages. Following such passages the loss of titer in tissue culture transfers decreased more slowly suggesting that the rabbit passages had altered the virus. This seems to be of the same nature as the changes noted in our experiments in which the adapted strain has been repeatedly restored to its initial pathogenicity by a single mouse passage, that pathogenicity being thereafter slowly lost by transfer in hamsters.

Our results also have something in common with those of Hirst (13). Hirst followed the mouse pathogenicity and egg infectivity of a strain of influenza virus while adapting it to mouse lung. Infectivity titers were uniformly high throughout, but pathogenicity titers slowly increased from generation to generation. In the case of mouse encephalomyelitis, mouse infectivity has likewise been relatively constant throughout the hamster generations but pathogenicity developed only after several transfers. Hirst suggested that several generations were necessary for the adapted, pathogenic strain to outgrow the parent, non-pathogenic strain.

Reference should also be made to the studies of Jungeblut, Feiner, and Sanders (14) who worked with Columbia SK virus. The adaptation of this agent to mice was effected through blind cotton rat passages. Its further adaptation to guinea pigs appeared to depend on the mouse passages used for transfer. Thus the third to twelfth mouse generations were not infective for guinea pigs while the seventieth to 162d generations were. This pattern was repeated in later generations and suggested fluctuations in the virulence of the agent so far as guinea pigs were concerned. Whether cyclic variations played a part in our experiments cannot be determined.

It is also of interest that hamster-adapted OT mouse encephalomyelitis virus is capable of inducing encephalitic signs which are rarely seen in mice inoculated with the OT type of virus but are so characteristic of the FA strain. The results suggest that the capacity to induce flaccid paralysis and encephalitis is inherent in both strains, and that these signs represent rather superficial

differences. Our experience with MM virus in hamsters supports this view since central nervous system inoculations of MM virus characteristically induce encephalitis, while the same suspensions cause flaccid paralysis if given intraperitoneally (4) or by gastric intubation.

Sanders observed (15) a change in the Lansing strain of poliomyelitis virus after repeated hamster passages. The titer of the virus rose rapidly when the agent was transferred to suckling hamsters. Adult and infant hamster strains were identical but were not neutralized by pooled human sera and were but irregularly neutralized by convalescent poliomyelitis monkey serum, all of which did neutralize the original mouse-adapted virus. It appeared "not impossible that our hamster virus represents a poliomyelitis-like agent of hamsters." The possibility cannot be dismissed. It seems an unlikely explanation of our own results since our hamster colony has been so carefully watched and tested for latent infections of this kind and because the virus did not change its behavior in the mouse. The neutralization tests are unfortunately incapable of distinguishing between the OT and FA strains. Sanders' results may possibly be explained as a change in antigenic pattern comparable to those produced by Hirst in influenza viruses. Whether such changes occur in mouse encephalomyelitis viruses is not known but relationship between them and poliomyelitis virus has been reported (16).

The short incubation period in hamsters may be due to species or to a difference in the immune status of the host, since the Albany mice are almost uniformly infected with the OT strain of encephalomyelitis virus and develop humoral antibodies while laboratory-bred hamsters are uninfected. If this is the correct explanation, the observations would be comparable to those of Schaeffer and Muckenfuss (17) who observed a prolongation of the incubation period in monkey poliomyelitis when the virus was mixed with immune serum. The mean incubation period following inoculation with virus-non-immune-serum mixtures was 8.3 days and with virus-immune-serum mixtures, 14.3 days.

CONCLUSIONS

The OT strain of mouse encephalomyelitis virus induces an inapparent infection in suckling hamsters associated with lesions of the central nervous system and skeletal muscles. The virus increases in pathogenicity after alternating mouse-hamster transfers and then induces both paralysis and encephalitis. Pathogenicity is lost through serial hamster passages but is restored by a single mouse transfer.

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